REMARKS

Claims 21, 23-29, and 31-37 are pending in this application. Claims 21, 23, 24, 26-29, 32, 33, 35, and 36 are amended to more particularly define the subject matter of the invention. Support for these amendments is found in the specification as filed at, for example, page 1, lines 17-18; page 6, lines 19-25; and page 12, lines 9-12.

REJECTIONS UNDER 35 U.S.C. § 112, FIRST PARAGRAPH

Enablement

The Examiner has maintained the rejection of claims 23, 24, 28-29, 32-33, 35, and 36 under 35 U.S.C. § 112, first paragraph alleging that the claims are not enabled. Specifically, the Examiner objects to the language "bone and/or cartilage inducing factor" and contends that the specification does not provide enablement for all bone or cartilage inducing factors or all BMPs, as broadly claimed. The Examiner contends that some of these factors have distinct biological activities that are sometimes opposite of one another (Vukicevic et al.; Massague; U.S. Patent 5,194,596; Benjamin et al.). The Examiner further alleges that the specification fails to teach the skilled artisan how to use all members of the TGF-β superfamily, all BMPs, and/or GDFs in the claimed invention without resorting to undue experimentation.

Applicants believe that the Examiner has misunderstood and consequently mischaracterized the arguments made in response to the Office Action dated June 25, 2005. Therefore, Applicants disagree with and traverse the statements made by the Examiner in support of this rejection.

Moreover, Applicants submit that the rejection is unwarranted and that the specification fully enables the claimed invention. The claims recite administration of a genus of proteins defined by well known structural characteristics, i.e., structural characteristics that are common to all member of the TFG-β superfamily (claims 23, 28, 32, and 35) or bone morphogenetic proteins (BMP) (claims 24-25, 29, 31, 33-34, and 36-37). The proteins are further defined by specific functional characteristics, i.e., the ability to induce the formation of cartilage tissue. Thus, only compounds that possess the specified structure and function are encompassed by the claims.

Methods for determining whether a BMP or other TFG-β superfamily protein is capable of inducing the formation of cartilage tissue are well known to those of skill in the art. For example, Wang et al., "Purification and Characterization of Other Distinct Bone-inducing Factors," *Proc. Natl. Acad. Sci. USA* 85:9484-9488 (1988) (copy enclosed) describes an *in vivo* cartilage and bone formation assay that was available well before the filing date of the application. Moreover, working examples of TFG-β superfamily members which fall within the claims are provided in the application. It would not require undue experimentation to determine whether any particular TFG-β superfamily protein or BMP is capable of inducing the formation of cartilage tissue. Nothing more is required to enable the claims.

Accordingly, Applicants respectfully request that the rejection be withdrawn.

Written Description

The Examiner has maintained the rejection of claims 23, 24, 28-29, 32-33, 35, and 36 as allegedly failing to comply with the written description requirement of 35 U.S.C. § 112, first paragraph. Specifically, the Examiner alleges that the structural and functional features required for describing a genus such as a TGF-β superfamily member or a BMP are not provided. Applicants traverse.

The rejected claims are directed to administering a TGF-β superfamily protein or a BMP that is capable of inducing the formation of cartilage tissue in combination with non-tissue culture expanded CD105+ cells isolated from bone marrow. The specification clearly describes the administration of a TGF-β superfamily protein or a BMP (e.g., at page 6, line 19 to page 7, line 6). The TGF-β superfamily proteins and BMPs are well known, art recognized, genuses of proteins that have been extensively studied and characterized by structure and function. See, e.g., Ozkaynak et al., "Osteogenic Protein-2 A New Member of the Transforming Growth Factor-β Superfamily Expressed Early in Embryogenesis," *J. Biol. Chem.* 267(35): 25220-7 (1992); Ducy and Karsenty, "The Family of Bone Morphogenetic Proteins," *Kidney International* 57:2207-14 (2000); and Wozney, "The Bone Morphogenetic Protein Family and Osteogenesis," *Molecular Reproduction and Development* 32:160-7 (1992) (copies enclosed).

The specification also clearly indicates that induction of cartilage tissue is a key feature of these proteins (e.g., at page 1, lines 11-12 and lines 18-19; page 6, lines 5-

10; page 11, lines 10-13). Only members of the recited genus of proteins that are capable of inducing cartilage tissue fall within the claims. This functional characteristic, like the structural characteristics of the TGF-β superfamily of proteins, is well known and understood by those of skill in the art. Together, the recitation of well known structural and functional characteristics of the encompassed proteins is sufficient description to meet the written description requirement of 35 U.S.C. § 112, first paragraph.

Moreover, the invention here does not lie in the discovery of that TGF-β superfamily proteins or BMPs are capable of inducing cartilage formation. As already discussed, these proteins are known in the art or can be identified using well known techniques without undue experimentation. Instead, the present invention lies in the discovery that non-tissue culture expanded CD105+ cells isolated from bone marrow may be administered to induce chondrogenesis. As a result, the Examiner's contention that the structural and functional features of every BMP or TGF-β protein must be fully presented in the application is an inappropriate generalization. See, *Capon v. Eshhar*, 418 F.3d 1349, 1358 (2005) ("When the prior art includes the [structural] information, precedent does not set a per se rule that the information must be determined afresh.")

Applicants respectfully submit that adequate description of TGF-β superfamily proteins or BMPs capable of inducing formation of cartilage tissue for combination with non-tissue culture expanded CD105+ cells isolated from bone marrow is provided by the specification. Accordingly, Applicants request that the rejection be withdrawn.

REJECTION UNDER 35 U.S.C. § 102

Applicants thank the Examiner for withdrawing previous rejections under 35 U.S.C. § 102(a).

Claims 21, 23-29, and 31-37 stand rejected under 35 U.S.C. § 102(e) as allegedly anticipated by U.S. Patent No. 6,761,887 (the '887 patent), which was also published as PCT publication WO 00/29552 on May 25, 2000. Applicants respectfully submit that the '887 patent is not prior art and therefore, this rejection is improper and must be withdrawn.

Patents issued directly or indirectly from international applications filed before November 29, 2000 may only be used as prior art based on the provisions of 35 U.S.C. § 102(e) in effect before November 29, 2000. Thus, the § 102(e) prior art date of such a patent is the earliest of the date of compliance with 35 U.S.C. § 371(c) (1), (2), and (4), or the filing date of the later-filed U.S. continuing application that claimed the benefit of the international application. Manual of Patent Examining Procedure, § 706.02(a)II(B).

The '887 patent has a 35 U.S.C. § 371(c)(1), (2), (4) date of June 21, 2001, as indicated on the face of the patent. This date is later than the present application's priority date of February 23, 2001. Therefore, the '887 patent is not a § 102(e) prior art reference and the Examiner should withdraw this rejection of claims 21, 23-24, 26-28, 32-33, and 35-36.

Claim 21, 23-24, 32-33, and 35-36 stand rejected under 35 U.S.C. § 102(e) as allegedly anticipated by U.S. Patent No. 6,835,377 (the '377 patent), which has an effective filing date of May 13, 1998. The Examiner contends that the '377 patent refers

to methods for regenerating articular cartilage and treating a cartilage defect resulting from osteoarthritis, comprising administering human mesenchymal stem cells to a host in need thereof. The Examiner further contends that the '377 patent teaches BMPs as molecules known to be involved with chondrogenesis. Applicants traverse this rejection for at least the following reasons.

Applicants' claims recite methods for inducing chondrogenesis using non-tissue culture expanded CD105+ cells. The methods of claims 21 and 26-28 may only be practiced with non-tissue culture expanded cells, while the '377 patent only describes tissue culture expanded cells. Applicants' invention provides the first description of the use of non-tissue culture expanded cells for inducing chondrogenesis.

There is a significant difference between a population of cells that have undergone rounds of tissue culture and a population of primary cells isolated from a patient. Cultured cells are continually expanded to produce an almost infinite number of cells, and the selection of the cells by adhesion to cell culture plates tends to select for the most stable and long-living cells types. In contrast, primary, non-culture expanded cells are limited in number and in their ability to survive upon implantation. Accordingly, the use of culture expanded cells for the induction of chondrogenesis does not anticipate the use of non-expanded cells in the same methods. Thus, the '377 patent does not teach all the limitations of the amended claims and Applicants respectfully request that this rejection be withdrawn.

REJECTION UNDER 35 U.S.C. § 103

The Examiner has rejected claims 21, 23-29, and 31-37 under 35 U.S.C. § 103(a) as allegedly unpatentable over U.S. Patent No. 6,835,377 (Goldberg *et al.*, U.S. 2002/0110544, claiming priority to May 13, 1998, the '377 patent) in view of Barbara *et al.* for the reasons stated at pages 9-10 of the Office Action. Applicants respectfully traverse this rejection for at least the following reasons.

The Examiner contends that it would have been obvious to the person of ordinary skill in the art to combine the teachings of the '377 patent with the teachings of Barbara *et al.*, to include BMP-2 as a factor that would induce chondrogenesis, because BMP-2 has been shown to interact with endoglin (CD105). The Examiner concludes that the skilled artisan—knowing that BMP-2 was involved in controlling processes such as embryogenesis, organogenesis, morphogenesis of tissues like bone and cartilage, wound repair, and hematopoieses—would have been motivated to include BMP-2 in a method for inducing chondrogenesis because BMP-2 has been shown to interact with endoglin (CD105) in a specific, but transient manner, thus permitting regulated chondrogenesis.

To establish a prima facie case of obviousness, the Examiner must establish that the cited art (alone, combined, or with obvious modification) teaches all the limitations of the claims. The Examiner must also prove that a motivation to combine the teachings of two references, or to modify the cited art, is present in the art or in the knowledge of one of skill in the art as of the priority date of the application. Finally, the Examiner must

establish that the cited art provided the skilled artisan with a reasonable expectation of success in practicing the claimed invention. M.P.E.P. § 2143.

As set forth above, the '377 patent does not teach all the limitations of the claims. At best, it only discloses or suggests the use of tissue culture expanded cells. Barbara et al. provides no remedy to the deficiency of the '377 patent, merely teaching that BMP-2 interacts with CD105. In contrast to the combined teachings of the '377 patent and Barbara et al., Applicants' claims are restricted to the use of non-tissue culture expanded CD105+ cells. Accordingly, these references do not teach or suggest all the limitations of Applicants' claims. Neither reference provides any motivation to replace the tissue culture expanded cells of the '377 patent with the non-culture expanded cells of the claimed invention.

Arguing in the alternative, even if the references did provide a motivation to use non-culture expanded cells, neither reference provides a reasonable expectation of success in achieving the induction of chondrogenesis by administration of non-tissue culture expanded cells. Instead, the '377 patent only describes the use of tissue culture expanded mesenchymal cells in chonrogenesis. For one skilled in the art to have any reasonable expectation of success in inducing chondrogenesis with non-culture expanded cells, several characteristics of the cells would need to be shown. First, the cells need to express BMP receptors. Second, the cells need to be capable of expressing chondrogenic genes, like Sox-9. Finally, the cells need to be able to live long enough in their non-expanded state to induce the desired chondrogenic effects.

The '377 patent does not even suggest that the cells described in that publication exhibit any of these characteristics, and, again, Barbara et al. does not cure this deficiency.

Instead, it is only with Applicants' disclosure that one skilled in the art would understand that the non-expanded CD105+ cells actually have the ability to induce chondrogenesis. Before Applicants' disclosure, one skilled in the art would have no way of knowing that these cells would have this ability. Accordingly, neither the '377 patent nor Barbara et al. provide one skilled in the art with a reasonable expectation of success in practicing the claimed invention.

Additionally, Applicants' invention has several unexpected advantages over the methods of the '377 patent. These advantages are described in Applicants' specification at page 8, lines 3-19 and page 18, lines 1-16. As discussed above, the mesenchymal stem cells disclosed in the '377 patent were tissue culture expanded and selected by adherence to plastic culture flasks. This adherence selection does not correlate with chondrogenic potential, whereas Applicants have demonstrated that the presence of CD105 on the cell surface does correlate with chondrogenic potential. Second, by immunoselecting CD105+ cells, the claimed invention avoids the deficiencies of a tissue culture expansion procedure, such as the likelihood of discarding potentially important cells based on their inability to adhere to tissue culture flask. Third, culture expansion may alter the cell surface characteristics of the cells, rendering them immunogenic to the host, while non-tissue culture expanded cells have

no opportunity to alter surface protein expression patterns. And finally, the use of non-tissue culture expanded cells eliminates the time-consuming, expensive, and laborious process of multiple steps of tissue culture expansion.

The teachings of the '377 patent are insufficient for establishing a case of obviousness, and the addition of the disclosure of Barbara et al. will not lead one of skill in the art any closer to the claimed invention. Therefore, Applicants respectfully request that the rejection of claims 21, 23-29, and 31-37 as allegedly obvious be withdrawn.

In view of the forgoing amendments and remarks, Applicants respectfully request reconsideration and reexamination of this application, and the timely allowance of the pending claims.

Please grant any extension of time required to enter this response and charge any additional required fees to deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: April 10, 2006

By: Elizabeth Mathiesen
Elizabeth E. Mathiesen
Reg. No. 54,696

Attachments:

- *Capon v. Eshhar*, 418 F.3d 1349 (2005)
- Wang et al., *Proc. Natl. Acad. Sci. USA* 85:9484-8 (1988)
- Ozkaynak et al., *J. Biol. Chem.* 267(35):25220-7 (1992)
- Ducy and Karsenty, *Kidney International* 57:2207-14 (2000)
- Wozney, Molecular *Reproduction and Development* 32:160-7 (1992)

MOLECULAR REPRODUCTION AND DEVELOPMENT 32:160–167 (1992)

The Bone Morphogenetic Protein Family and Osteogenesis

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ABSTRACT The BMPs (bone morphogenetic proteins) are a group of related proteins originally identified by their presence in bone-inductive extracts of demineralized bone. By molecular cloning, at least six related members of this family have been identified and are called BMP-2 through BMP-7. These molecules are part of the TGF-beta superfamily, based on primary amino acid sequence homology, including the absolute conservation of seven cysteine residues between the TGF-betas and the BMPs. The BMPs can be divided into subgroups with BMP-2 and BMP-4 being 92% identical, and BMP-5, BMP-6, and BMP-7 being an average of about 90% identical. To examine the individual activities of these molecules, we are producing each BMP in a mammalian expression system. In this system, each BMP is synthesized as a precursor peptide, which is glycosylated, processed to the mature peptide, and secreted as a homodimer. These reagents have been used to demonstrate that single molecules, such as BMP-2, are capable of inducing the formation of new cartilage and bone when implanted ectopically in a rodent assay system. Whether each of the BMPs possesses the same inductive activities in an animal is the subject of ongoing research. Based on the chondrogenic and osteogenic abilities of the BMPs in the adult animal, the expression of the mRNAs for the BMPs has been examined in the development of the embryonic skeleton by in situ hybridization. These studies demonstrate that the BMP mRNAs are spatially and temporally expressed appropriately for the proteins involved in the induction and development of cartilage and bone in the embryonic limb bud. Furthermore, primary preparations of limb bud cells respond to BMP-2, as do several cell lines of the osteoblastic lineage. In addition to expression in the skeletal system, various of the BMP mRNAs are expressed in distinct tissues, suggesting additional roles during development.

Key Words: BMP, TGF-beta superfamily, Bone

INTRODUCTION

Similar to the process described by Anita Roberts for TGF-beta, the discovery of the Bone Morphogenetic Proteins (BMPs) came from a search for the molecules responsible for a particular activity. In this case, it had been known for about 30 years that protein extracts from bone implanted into animals at non-bone sites will induce the formation of new cartilage and bone tissues (Urist, 1965). In this system, the sequence of events recapitulates the process of bone formation seen during embryonic long bone development (Urist et al., 1979; Reddi, 1981). This process is known as endochondral ossification, in which you observe a cartilage intermediate rather than intramembranous bone formation where bone is formed directly from mesenchyme. The identification of these osteoinductive molecules would allow their use as therapeutic treatments for a variety of bone defects, including nonunions, fractures, and periodontal disease.

Purification of Bovine BMP and Cloning of the Human BMPs

BMP activity was purified from bovine bone using ectopic bone induction in rats as the assay system (Sampath and Reddi, 1981), which is a reconstitution assay. Bone can be seen as consisting of three different components: 1) a mineral component which gives it its structural integrity, 2) a collagenous matrix component, and 3) a growth factor component which contains the BMP activity. The growth factor component can be extracted after the bone has been demineralized. To follow the BMP through its purification, the protein was assayed by reconstituting the fractions with rat bone collagenous matrix from which the endogenous BMP activity has been removed, and testing the effect of these reconstituted fractions by implantation subcutaneously in a rat. The implants were left in the animal for 7–14 days and then removed and examined histologically for the presence of newly formed cartilage and bone tissue. Thus, this assay system, which defines the BMP activity, is the result of a complex series of cellular events. At early times the implant area is infiltrated by the undifferentiated cell types. By days 4–7 these undifferentiated mesenchymal cells differentiate into chondrocytes. The chondrocytes mature and calcify. Around day 10, the beginning of new bone formation is seen as the cartilage intermediate is removed. At very late times the tissue develops into normal remodeling bone tissue which is complete with osteoblasts in the process of forming bone and osteoclasts that are resorbing it.

Using this assay system, we purified BMP activity approximately 300,000-fold from bovine bone (Wang et

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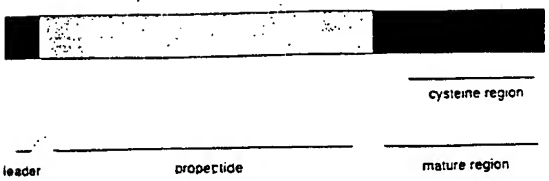

Protein	Additional names	Amino acid residues	Glycosylation sites mature
BMP-2	BMP-2A	396	1
BMP-4	BMP-2B	408	2
BMP-5		454	3
BMP-6	Vgr-1	513	3
BMP-7	OP-1	431	3
BMP-3	osteogenin	472	1

Fig. 1. Structure of the BMP proteins derived from cDNA clones. A generic BMP molecule is shown schematically with its secretory leader sequence, propeptide region, and carboxy-terminal mature region. Each of the BMPs contains N-linked glycosylation sites in both the propeptide and mature regions. The table below contains alternative nomenclature for the BMPs, the number of amino acids in the preprotein (primary translation products), and the number of potential N-linked glycosylation sites in the mature regions of the molecules.

al., 1988). While the yield is difficult to determine, there is probably about 20 μg of protein with bone inductive activity in about 10 kg of bovine bone. By assaying for BMP activity in proteins eluted from various gel electrophoresis systems, we determined that the BMP activity behaves in electrophoresis as a protein with a molecular weight of about 30,000 daltons, and the BMP protein is basic. However, gel electrophoresis of the 30,000 molecular weight protein under reducing conditions indicated the presence of multiple protein bands. We were not able to further purify the activity or determine which protein bands were responsible for the BMP activity because the activity is sensitive to reduction. Therefore, our strategy was to take the protein that we knew had BMP activity, digest it with trypsin, determine the sequences of the tryptic peptides, derive cDNA clones, express the cDNA clones, and determine which recombinant proteins in the mixture had osteoinductive activity.

From our cloning efforts we ended up with seven different proteins which we have named BMP-1 through BMP-7 (Wozney et al., 1988; Celeste et al., 1990). Six of the seven proteins are in the TGF-beta family (see Fig. 1). They are all secreted proteins with a hydrophobic leader sequence and a substantial propeptide region. Unlike TGF-beta, each of these proteins is glycosylated. These molecules are also known by alternative nomenclatures. For example, BMP-3 is the same as osteogenin (Luyten et al., 1989); BMP-6 is the human homologue of the murine Vgr-1 protein (Lyons et al., 1989; and BMP-7 has also been called OP-1 (osteogenic protein 1, Özkaynak et al., 1990).

The BMP Protein Family

The BMP proteins can be divided into subgroups based on the primary amino acid sequence in the mature regions of the molecule. BMP-2 and BMP-4 are quite closely related molecules, being 92% identical in the cysteine portion of the mature region. BMP-5, BMP-6, and BMP-7 form another subgroup with about 90% amino acid identity in the same region. These two subgroups are interrelated by about 60% amino acid identity, while BMP-3 is in a group of its own.

Comparing BMPs to other members of the TGF-beta superfamily, BMP-2, BMP-4, BMP-5, BMP-6, and BMP-7 are most closely related to *Drosophila decapentaplegic (dpp)*. Because BMP-2 and BMP-4 are so closely related to *dpp*, it is quite clear that these molecules are its mammalian homologues. BMP-5, BMP-6, and BMP-7 are probably the mammalian homologue to the newly discovered 60A or *Vgr*/60A gene. The next most closely related protein to the BMPs is Vg1, a protein of unknown function from *Xenopus laevis*. After Vg1, the next most closely related protein is activin-A. The BMPs are also distantly related to TGF-beta 1, 2, and 3.

When BMP-2 was expressed as a recombinant protein in CHO cells, the transfected cells secreted a variety of BMP-2 molecules, the most predominant being a homodimer of the mature molecule. In addition, the clipped propeptide region is found secreted into the culture medium. The BMP-2, BMP-4, and BMP-7 propeptides are secreted as monomers because they contain no cysteine residues to allow intermolecular crosslinks. We also observed small amounts of partially processed dimers of each of the BMPs.

Osteogenic Activity of BMP in the Rat Ectopic Assay

We first assayed the recombinant BMP proteins in the rat ectopic assay system to compare their activities with bone-derived BMP (Wang et al., 1990). Initially we used rat demineralized inactive bone matrix as a carrier for BMP-2, placed rhBMP-2 combined with this matrix subcutaneously into the rat, and left the implants for various times before examining them histologically. In the control implants, the carrier matrix particles elicited migration of occasional undifferentiated mesenchymal cells into the site. The inclusion of BMP-2 resulted in much more cell invasion as well as recognizable chondrogenesis by 5 days. At 7 days there was some cartilage remaining in the implants, some new bone formation, and the matrix was being resorbed. After about 21 days we were left with a small ossicle of mineralized bone, a layer of osteoblasts laying down bone, and a mature fatty marrow. From these studies it was evident that BMP-2 is sufficient to induce the formation of bone in vivo, and that the one molecular species rhBMP-2, has all the activity of bone-derived BMP in this assay system.

To further quantitate the action of BMP-2 we established a scoring system on a sliding scale of 0–5 in

which the score 0 indicates no cartilage or bone present, and a score of 5 is given when the entire implant is composed of cartilage or bone. Using this scoring system, we did a dose-response and time course study. These results showed that cartilage formed at earlier times and then was replaced by bone at later times. It was possible to decrease the time required to observe bone formation by increasing the amount of rhBMP-2 in the matrix. High concentrations of rhBMP-2 resulted in concurrent cartilage and bone formation at early times after protein implantation.

A comparison of the primary sequences of BMP-2 and BMP-4 shows that these proteins are almost identical in the cysteine domain. However, the two proteins are quite different in the amino terminal domain despite the fact that they are both very basic in this region. Both proteins are active in the rat ectopic system (Hammonds et al., 1991), although addition of about twice as much BMP-4 was required for the same amount of bone formation as seen with doses of BMP-2. The time course of BMP-4-induced bone formation was slightly slower than for BMP-2.

BMP-5 also has the same osteoinductive effect as BMP-2 and BMP-4, but the time course of the osteoinductive response is significantly delayed when compared with that of BMP-2 (Cox et al., 1991; D'Alessandro et al., 1991). With BMP-5 implantation there is no observed bone formation until day 7. Furthermore, significantly more BMP-5 than BMP-2 is needed to observe the same amount of bone formation (Fig. 2). The same levels of bone formation as achieved by BMP-2 are never achieved with BMP-5 at either a comparable dose or a comparable time.

To summarize, bone-derived BMP activity is due to a set of proteins related to TGF-beta. In our hands we would say it is due to the proteins BMP-2 through BMP-7. Others have reported purifications where they observe activity with a combination of BMP-2 and BMP-7 (Sampath et al., 1990, 1991), or that BMP-3 is the sole BMP activity (Luyten et al., 1989). Individual proteins of this family of BMP molecules are sufficient alone for induction of bone formation in vivo. This has been demonstrated for BMP-2, BMP-4, BMP-5, and BMP-7. However, conclusive data has not yet been reported for BMP-3.

Induction of Bone in the Segmental Defect Model

We have initiated some studies to determine whether BMP proteins, in particular BMP-2, will be therapeutically useful in the human. Specifically, we wanted to answer the following questions: First, will rhBMP-2 induce bone formation at a bony site and produce new bone that will integrate with the preexisting bone to form a functional union? Second, will rhBMP-2 be able to induce bone formation in higher animals? For example, while the data above demonstrates that the BMPs will induce bone at an ectopic site in a rat, it is known that the rate of bone formation in rats is significantly higher than in humans.

One study was done using a rat femoral defect model in collaboration with Alan Yasko and Joseph Lane at the Hospital for Special Surgery in New York (Yasko et al., 1991). In this model a 5 mm femoral defect was created and fixed using a polyethylene plate attached with Kirshner wires; rhBMP-2 was implanted in the defect in a bed of inactive bone matrix. The experimental groups in this study were treated with a low or high dose of recombinant BMP-2. In all the studies the carrier system was allogeneic, guanidine-extracted bone matrix. The matrix alone was used as the control. Bone growth was evaluated by three criteria: 1) weekly autoradiographs, 2) histology at the end of the study, and 3) biomechanical studies. Without treatment, a defect of this size will proceed to become a nonunion in 100% of cases. Thus, in the controls, there was a small amount of growth at both ends of the defect but insufficient osteoconduction to fill the gap. By 4.5 weeks, in defects treated with the higher doses of rhBMP-2 there was already sufficient bone formation to be defined as a union by an orthopedic surgeon examining the radiographs. The results of biomechanical studies in which the femurs of four animals implanted with rhBMP-2 were torque-tested to determine the strength of the bone showed that the bone was quite strong compared to the untreated femurs. The presence of the holes through which the Kirshner wires were threaded weakened the BMP-treated femurs such that they could not be as strong as the untreated femur.

In a second study, sheep were used as the experimental animal (Gerhart et al., 1991). A similar experimental design was used as for the rat in which BMP adhered to matrix or matrix alone was implanted in a 2.5 cm defect in the sheep femur held together by a single metal plate. This study was done in collaboration with Dr. Tobin Gerhart and Dr. Carl Kirker-Head at Tufts Veterinary School. Eleven of the twelve sheep completed the study. In one sheep there was a failure of the fixation plate. The matrix control failed to heal and by the end of the study the region was quite grossly mobile and had developed a pseudoarthrosis. We also performed autologous bone grafting as a positive control. This is the current preferred therapy that would be used to treat a defect like this in humans. The autologous graft and the BMP-2-treated defect showed union by 12 weeks. In these animals the implantation sites of the femurs were grossly quite rigid. The results of biomechanical studies showed that the BMP-2-treated bone and the autologous graft were comparable to the contralateral control. By contrast, the bone with no implant or the bone treated with matrix alone showed no bone union. Histologically a large amount of bone formation could be observed in the BMP-2-treated defect with some remodeling already evident in which a cortex with a marrow cavity was being produced.

Our third study was done in collaboration with Dr. Dean Toriumi and used a dog mandibular defect model (Toriumi et al., 1991). The dog is considered to have bones that most closely approximate those of humans in size and remodeling sequence. In addition, this site pro-

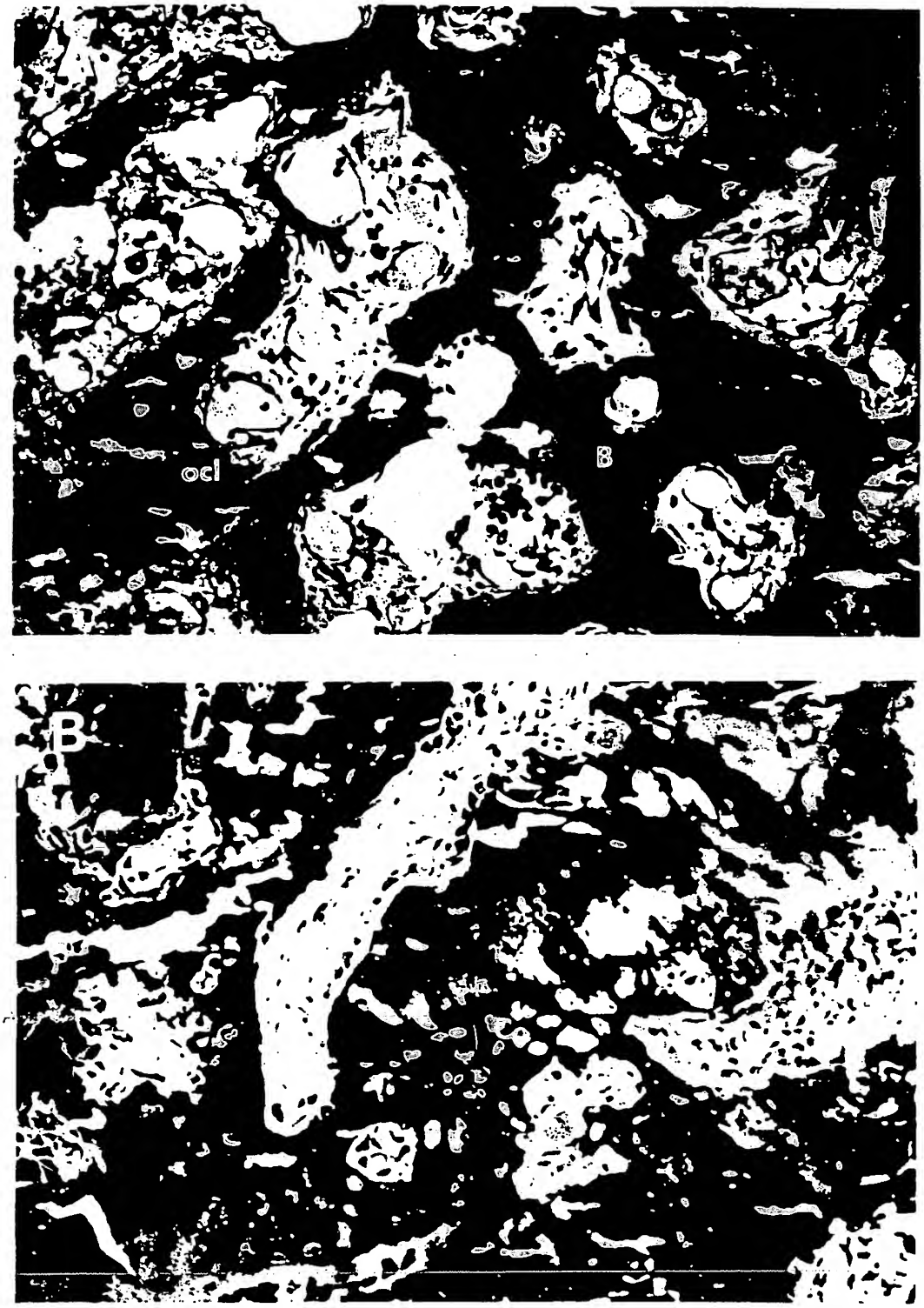

Fig. 2. Comparison of activities of rhBMP-2 and rhBMP-5: 10 μg of rhBMP-2 (A) or rhBMP-5 (B) were implanted subcutaneously in rats for 15 days. Implants were removed and processed for histology. C, calcified cartilage; ob, osteoblasts; M, unresorbed carrier matrix particles; ocl, osteoclast; V, blood vessel; mw, bone marrow; B, newly formed bone.

vides information on the efficacy of BMP-2 bone induction in a bone which is formed through the intramembranous sequence during embryogenesis, rather than the endochondral process that we associate with long bones. Three centimeter defects were made in dog mandibles. The mandibles were fixed with a metal plate, and then implanted with matrix alone as a negative control, matrix impregnated with BMP, or left empty. Filling of the defect with matrix alone resulted in fibrosis in the defect area, the same as leaving the defect empty. With BMP-2, the defect was totally filled with bone after 3 months. By this time the bone was already significantly remodeled.

To summarize the results of these studies, we have found that rhBMP-2 induces bone in all the models we investigated; in the rat ectopic bone formation assay and in the rat, sheep, and dog segmental defect models. It is interesting that the amount of BMP-2 needed and the time required to produce bone is similar in all these animals even though the sizes of the animals and their metabolic rates are significantly different. The rate-limiting parameter may be the volume of bone to be replaced; that is, the amount of time it takes the responsive cells to penetrate through the implant site and respond to the BMP-2.

The events observed in response to BMP in vivo are quite complex. It is possible that the function of the BMPs is to induce the initial step of the differentiation of mesenchymal cells into chondrocytes and then in vivo other growth factors, cells, and processes become involved to yield the cascade of bone formation. There is also some circumstantial evidence that BMP-2 may affect multiple stages in the bone formation process. In most studies the sequence of events observed in response to BMP implantation in vivo was differentiation of mesenchyme, chondrogenesis, hypertrophy, maturation of the cartilage, removal of hypertrophic cartilage, and then osteogenesis. However, with large amounts of BMP, osteogenesis can be seen concurrently with chondrogenesis. We were therefore interested to determine whether rhBMP-2 could affect the osteogenic phenotype of cultured cells.

The calvarial-derived, multipotential C26 cells are capable of differentiating into osteoblasts, adipocytes or muscle cells. In a collaboration with Dr. Suda and Dr. Yamaguchi, the effects of rhBMP-2 on these cells were examined (Yamaguchi et al., 1991). At large doses of rhBMP-2, we observed induction of alkaline phosphatase and a cAMP response to PTH, both markers of osteoblasts. In addition, the ability of these cells to differentiate into muscle decreased after exposure to rhBMP-2, as measured by the number of desmin-positive cells. Also, rhBMP-2 strikingly induced the expression of BGP mRNA. BGP is also called bone gla protein or osteocalcin and is probably the one specific marker of the mature osteoblast phenotype. The induction of osteocalcin was enhanced by the presence of 1,25 dihydroxy-vitamin D3.

Another osteoprogenitor cell compartment is the bone marrow stroma. W-20 cells are a multipotent mouse bone marrow stromal cell that is able to differentiate into osteogenic cells and adipocytes. Upon treatment with rhBMP-2, W-20 cells increased the expression of alkaline phosphatase (Thies et al., 1992). We have investigated numerous factors in this assay and have found no other factor or hormone which has been implicated in bone formation that will induce W-20 cell alkaline phosphatase production. In contrast, TGF-beta appeared to slightly inhibit alkaline expression by W-20 cells. BMP-5 also increased the production of alkaline phosphatase by W-20 cells, but the dose response was shifted such that more BMP-5 than BMP-2 was required for comparable enzyme induction. These results paralleled our observations on bone induction in vivo, i.e., in both cases, more BMP-5 than BMP-2 was required for a comparable effect. On the other hand, BMP-4 was more active than BMP-2 in the cell cultures, but slightly less active than BMP-2 in vivo. Trypsin-treated BMP-2 had a dose response that exactly overlaid that of BMP-4. This limited trypsin digestionof BMP-2 resulted in loss of the N-terminal region in which exists the major differences in sequence between BMP-2 and BMP-4, suggesting that the N-terminus of BMP-2 modulates its action. The N-terminus may inhibit the activity of BMP-2 by increasing its interaction with nonsaturable binding sites on the cell's surface or in the extracellular matrix, such that the concentration of BMP-2 available to its receptors is lower than its actual concentration.

Recently reported by Sampath and his colleagues are some studies of the responses of primary calvarial osteoblast-like cells to BMP-7 (also called OP-1; Knutsen et al., 1991). These cells, which are probably a mixture of osteoblast progenitors at different stages of differentiation, responded to BMP-7 with increases in collagen production, proliferation, and increases in various osteoblast characteristics such as alkaline phosphatase activity, PTH-stimulated cAMP production, osteocalcin synthesis, and mineralized nodule formation (BMP-7 in combination with ascorbate and betaglycerolphosphate).

So, from all these studies of cultured cells, it seems that the BMPs can stimulate cells to differentiate into the osteoblastic phenotype or cause them to increase the expression of osteoblastic phenotypic markers.

The Role of BMPs in Embryogenesis

The first evidence for a role of BMPs in embryogenesis is the realization that BMP-2 and BMP-4 are the mammalian homologues of the *Drosophila dpp*. From the results of powerful genetic analyses of Drosophila we know that *dpp* is responsible for either delivering positional information or interpreting it. Therefore BMP-2 and/or BMP-4 may have similar roles in mammalian development. The second line of circumstantial evidence for their role in mammalian development comes from the results of in situ localization of the various mRNAs for the BMPs during murine embryogenesis (Rosen et al., 1989; Lyons et al., 1989, 1990; Jones et al., 1991). Bone morphogenetic proteins 2 and

Fig. 3. Localization of BMP-4 transcripts in the developing mouse limb bud. Sections of 10.5 dpc mouse embryos were hybridized to a BMP-4 RNA probe labeled with [^{35}S]. Localization is evident in the apical ectodermal ridge.

4 were both found in the apical ectodermal ridge of the limb-bud very early in development (Fig. 3). The apical ectodermal ridge is required for positional information in the developing limb bud. Later in development BMP-2 mRNA was found in the interdigital mesenchyme of the limb located between the regions where the cartilaginous condensations will occur. BMP-2 mRNA expression is also observed in prevertebrae and in the tooth bud. Although in one report BMP-2 and not BMP-4 was seen in the developing tooth bud (Jones et al., 1991), others have observed that BMP-4 is also expressed in the developing tooth but with a different spatial distribution than BMP-2. Later in development, BMP-2 and BMP-4 are found in the more mature perichondrium, periosteum, and in odontoblasts. We have never seen BMP-3 in developing limbs or the skeletal system until very late in development when it appears in the periosteum. We also know that BMP-6 (Vgr-1) is not seen early in the developing limb but is seen later in the hypertrophic cartilage. Thus, BMP-6 may have a significantly different role from BMP-2 or BMP-4 in the developing skeleton.

Along with areas of bone formation, the BMPs were also found in many other regions of the developing embryo. For example, BMP-2 is found in the heart and in whisker follicles. BMP-6 is found in brain, spinal cord, and skin. BMP-3 is found in the brain. In many of these regions various TGF-betas are also expressed.

Because of our interest in the developing limb and the role of BMPs in this process, we were interested in whether the BMPs could affect the development of limb bud cells. Therefore we have isolated cell lines from the limb buds by taking mouse embryos at various stages, removing the limb buds,and immortalizing the cells in the limb buds using a retroviral vector with a c-*myc* gene and a neomycin resistance gene as a selectable marker. The neomycin resistant cells were selected and examined for their phenotype and their growth responses to BMPs. Most of the work to date has been on cells derived from 13-day mouse embryos. The pool of cells responded to BMP-2 with a substantial increase in alkaline phosphatase, which could be potentiated by the addition of retinoic acid. The pool also responded to BMP-2 with an increase in bone gla protein expression (Rosen et al., 1991). After treatment with BMP-2, the pool of immortal limb bud cells formed nodules which could be stained with Alcian blue, a stain specific for cartilage extracellular matrix proteins. Therefore these cells were capable of forming cartilage nodules, a process that was significantly increased by the addition of BMP-2. In micromass cultures densely staining cartilage nodules could also be observed with the addition of BMP-2. Both the number of Alcian blue staining nodules and the incorporation of radiolabeled sulfate into proteoglycans can be used to quantitate the chondrogenic response to BMP-2. Using these methods, there is both a dose and time dependent increase in expression of the chondrogenic phenotypes in response to BMP-2.

We have also derived individual clones from this pool of cells and they display a variety of phenotypes. For example, clone number 5 responded to BMP-2 with at least a 10-fold increase in alkaline phosphatase but showed no response to TGF-beta-1. This clone also responded with an increase in cAMP when treated with PTH. Thus, one can derive cells from embryonic mouse limb buds at times as early as 13 days of development that express markers of the osteoblast lineage.

To summarize what we know about the activities of rhBMP-2 in cultured cells: it increases expression of markers of the osteoblast phenotype in the MC3T3E1 and the C26 cell lines. The C20 cells, which represent more differentiated osteoblasts, show no increase in alkaline phosphatase but some increase in PTH-stimulated cAMP production. From these results it seems that the cells which are more responsive to the BMPs are the earlier osteoprogenitor cells rather than the more differentiated osteoblasts. The activity of BMP-2 on W-20 cells derived from the bone marrow stromal environment is to increase alkaline phosphatase, PTH-

stimulated cAMP production, and osteocalcin synthesis but not to increase proliferation. In the embryonic 10T1/2 cell line it has been reported that alkaline phosphatase and the PTH response increases after BMP treatment. Finally, the BMPs increase several parameters and characteristics of the chondrogenic lineage in the immortal embryonic limb bud cells.

In summary, BMP-2 activates cells that participate in bone repair. This has been demonstrated in vivo in a variety of species, as well as in cultured cells from both the osteoblastic and chondroblastic lineages. In adult animals, rhBMP-2 is capable of inducing the formation of new bone, and thus can successfully treat large bony defects. The BMPs can also increase the differentiated state of primary embryonic cells, indicating that the BMPs are involved in the development of cartilage and bone during embryogenesis.

ACKNOWLEDGMENTS

A large number of people at Genetics Institute have contributed to the understanding of the BMPs in bone development. I would like to particularly acknowledge Vicki Rosen and her cell biology group, as well as Liz Wang and her biochemistry group. Karen Cox for the histological analysis of rhBMP-2 and rhBMP-5 (Fig. 2), and Joanna Capparella for the in situ hybridization analysis (Fig. 3).

REFERENCES

Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM (1990): Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci USA 87:9843–9847.

Cox K, Holtrop M, D'Alessandro JS, Wang EA, Wozney JM, Rosen V (1991): Histological and ultrastructural comparison of the in vivo activities of rhBMP-2 and rhBMP-5. J Bone Min Res 6:S155.

D'Alessandro JS, Cox KA, Israel DI, LaPan P, Moutsatsos IK, Nove J, Rosen V, Ryan MC, Wozney JM, Wang EA (1991): Purification, characterization and activities of recombinant bone morphogenetic protein 5. J Bone Min Res 6:S153.

Gerhart TN, Kirker-Head CA, Kriz MJ, Hipp JA, Rosen V, Schelling S, Wozney JM, Wang EA (1991): Healing of large mid-femoral segmental defects in sheep using recombinant human bone morphogenetic protein (BMP-2). Trans Orthop Res Soc 16:172.

Hammonds RG Jr, Schwall R, Dudley A, Berkemeier L, Lai C, Lee J, Cunningham N, Reddi AH, Wood WI, Mason AJ (1991): Bone-inducing activity of mature BMP-2b produced from a hybrid BMP-2a 2b precursor. Mol Endocrinol 5:149–155.

Jones CM, Lyons KM, Hogan BLM (1991): Involvement of Bone Morphogenetic Protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. Development 111:531–542.

Kuntsen R, Mohan S, Wergedal J, Sampath K, Baylink DJ (1991): Osteogenic protein-1 (OP-1) stimulates proliferation and differentiation of human bone cells (HBC) in vitro. J Bone Min Res 6:S141.

Luyten FP, Cunningham NS, Ma S, Muthukumaran N, Hammonds RG, Nevins WB, Wood WI, Reddi AH (1989): Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. J Biol Chem 264:13377–13380.

Lyons K, Graycar JL, Lee A, Hashmi S, Lindquist PB, Chen EY, Hogan BLM, Derynck R (1989): Vgr-1, a mammalian gene related to Xenopus Vg-1, is a member of the transforming growth factor β gene superfamily. Proc Natl Acad Sci USA 86:4554–4558.

Lyons KM, Pelton RW, Hogan BLM (1989): Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-β-like genes coordinately regulate aspects of embryonic development. Genes Dev 3:1657–1668.

Lyons KM, Pelton RW, Hogan BLM (1990): Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for Bone Morphogenetic Protein-2A (BMP-2A). Development 109:833–844.

Ozkaynak E, Rueger DC, Drier EA, Corbett C, Ridge RJ, Sampath TK, Oppermann H (1990): OP-1 cDNA encodes an osteogenic protein in the TGF-β family. EMBO J 9:2085–2093.

Reddi AH (1981): Cell biology and biochemistry of endochondral bone development. Collagen Rel Res 1:209–226.

Rosen V, Bauduy M, McQuaid D, Donaldson D, Thies S, Wozney J (1991): Expression of osteoblast-like phenotype in mouse embryo limb bud cell lines cultured in BMP-2 and retinoic acid. Proc Endocrine Soc 73:57.

Rosen V, Wozney JM, Wang EA, Cordes P, Celeste A, McQuaid D, Kurtzberg L (1989): Purification and molecular cloning of a novel group of BMPs and localization of BMP mRNA in developing bone. Connec Tissue Res 20:313–319.

Sampath TK, Coughlin JE, Whetsone RM, Banach D, Corbett C, Ridge RJ, Ozkaynak E, Oppermann H, Rueger DC (1990): Bovine osteogenic protein is composed of dimers of OP-1 and BMP-2A, two members of the transforming growth factor-β superfamily. J Biol Chem 265:13198–13205.

Sampath TK, Ozkaynak E, Jones WK, Sasak H, Tucker R, Tucker M, Kusmik W, Lightholder J, Pang R, Corbett C, Oppermann H, Rueger DC (1991): Recombinant human osteogenic protein (hOP-1) induces new bone formation with a specific activity comparable to that of natural bovine osteogenic protein. J Bone Min Res 6:S155.

Sampath TK, Reddi AH (1981): Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. Proc Natl Acad Sci USA 78:7599–7603.

Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V (1992): Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology: 130:1318–1324.

Toriumi DM, Kotler HS, Luxenberg DP, Holtrop ME, Wang EA (1991): Mandibular reconstruction with a recombinant bone-inducing factor. Arch Otolaryngol Head Neck Surg 117:1101–1112.

Urist MR (1965): Bone: Formation by autoinduction. Science 150:893–899.

Urist MR, Mikulski A, Lietze A (1979): Solubilized and insolubilized bone morphogenetic protein. Proc Natl Acad Sci USA 76:1828–1832.

Wang EA, Rosen V, Cordes P, Hewick RM, Kriz MJ, Luxenberg DP, Sibley BS, Wozney JM (1988): Purification and characterization of other distinct bone-inducing factors. Proc Natl Acad Sci USA 85:9484–9488.

Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel D, Hewick RM, Kerns K, LaPan P, Luxenberg DP, McQuaid D, Moutsatsos I, Nove J, Wozney JM (1990): Recombinant human bone morphogenetic protein induces bone formation. Proc Natl Acad Sci USA 87:2220–2224.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988): Novel regulators of bone formation: Molecular clones and activities. Science 242:1528–1534.

Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda T, Yoshiki S (1991): Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113:681–687.

Yasko AW, Lane JM, Fellinger EJ, Rosen V, Wang EA, Wozney JM, Gross JM, Glasser DB (1991): Recombinant BMP-2a bone induction in a rat orthotopic model. Trans Orthop Res Soc 16:410.

QUESTIONS AND ANSWERS

Q: *Can the bone matrix that you use be substituted by a synthetic polymer?*

A: That's a good question. Essentially all the studies that I discussed have been repeated using a defined collagenous or a synthetic matrix and BMP-2 works perfectly well with these matrices.

Q: *Have you observed any effect of TGF-beta-1 or TGF-beta-2 in vivo?*

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Biochemistry

Purification and characterization of other distinct bone-inducing factors

(bone morphogenetic protein/bone formation)

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ABSTRACT **We purified a factor that induces bone formation >300,000-fold from guanidinium chloride extracts of demineralized bone. Fifty nanograms of highly purified protein was active in an *in vivo* cartilage and bone-formation assay. The activity resided in a single gel band, corresponding to a molecular mass of ≈30 kDa, which yielded proteins of 30, 18, and 16 kDa on reduction. The partial amino acid sequence obtained from these proteins confirmed our identification of specific factors that induce new bone formation *in vivo*.**

Bone is a complex tissue that undergoes constant remodeling in response to changing physical demands. The signals that control resorption and formation, whether from humoral or localized growth and differentiation factors, extracellular matrix, or other presently unknown controls, require much further study. One approach to studying bone development is use of *in vivo* ectopic bone formation—the best characterized model of which is induction by demineralized bone implanted intramuscularly or subcutaneously. During this sequence of events (*i*) mesenchymal cells are seen to migrate into the implant, proliferate after several days, and condense in regions. (*ii*) Chondroblasts, believed to be derived from the early-appearing mesenchymal cells, form a cartilaginous template in the area of presumptive bone. (*iii*) At 10–14 days, the cartilage hypertrophies, and the cartilage extracellular matrix is vascularized by hematopoietic and endothelial cells. (*iv*) The cartilage is gradually removed and replaced by bone, and at the end of 21 days an ossicle of bone, complete with marrow, has been formed. This response is localized to the implant itself. The morphological but not temporal developmental sequence is the same as seen in embryonic endochondral bone formation and adult fracture repair (1–3).

This induction of the natural sequence of bone formation immediately suggested potential application for human therapeutics and for developmental studies. Thus began the search for a factor, or factors, named bone morphogenetic protein (BMP) by Urist (1), that could induce bone formation. BMP was characterized as an activity tightly bound to the matrix of demineralized bone and extractable by denaturing solvents (4). Implantation of protein itself was sufficient to induce bone, but reconstitution of the factor with a collagenous matrix (5, 6) or synthetic matrices (7, 8) enhanced sensitivity of the assay. Although purification and characterization have been hampered by the cumbersome *in vivo* assay, numerous reports have described osteoinductive factors (8–10). Additionally, many other growth factors, namely fibroblastic growth factor, platelet-derived growth factor, transforming growth factors β1 and β2, insulin-like growth factors I and II, and bone-derived growth factor, have been implicated in bone development by their presence in bone and their effect on cartilage and bone cells *in vitro*, although no direct osteoinductive role has yet been identified *in vivo* (for reviews, see refs. 11 and 12). We used the rat ectopic bone formation assay to further analyze BMP activity from bovine bone. We report here the purification of a discrete BMP, as distinguished by physical characteristics and extremely high specific activity.

MATERIALS AND METHODS

Materials. Ground bovine bone (20-120 mesh) was obtained from American Biomaterials (Plainsboro, N.J.) and N-Glycanase was obtained from Genzyme. DE-52 cellulose and CM-cellulose were obtained from Whatman; the hydroxyapatite was purchased from LKB; all other resins were obtained from Pharmacia. All chemicals were reagent grade except for the urea and guanidinium chloride, which were ultrapure.

Methods. *Biological assay.* The ectopic bone formation assay was done as described (5) with the following modifications. The protein to be assayed was equilibrated in a volatile solvent, generally 0.1% trifluoroacetic acid (CF_3COOH), and then mixed with 20 mg of demineralized, guanidinium chloride-extracted rat bone matrix. The material was frozen and lyophilized, and the powder was enclosed in no. 5 gelatin capsules. The capsules were implanted subcutaneously in the abdominal thoracic area of 21- to 49-day male Long–Evans rats and routinely removed at 7 days. Samples were processed for histological analysis, with 1-μm glycolmethacrylate sections stained with Von Kossa and acid fuschin or toluidine blue. Sections from implants were scored on a scale of 0–5 for the presence of new cartilage and bone. A cartilage score (c) of 5 indicates that >50% of the section was cartilage, a score of 4 indicates 40–50% cartilage, and a score of 3 indicates 30–40%, etc. A +/− indicates <5% cartilage, and the score by itself is not considered significant. (Scoring of illustrative samples is shown in Fig. 1.)

Purification. The initial extraction steps have been described (13, 14), the purification scheme is summarized in Table 1, and buffer conditions are detailed here. The dialyzed guanidinium chloride extract from 20 kg of bone was passed over a 3-liter DE-52 column equilibrated in 50 mM Tris/0.1 M NaCl/6 M urea, pH 7.2. The unbound fraction was then adsorbed to a 2-liter CM-cellulose column in 50 mM NaAc/50 mM NaCl/6 M urea, pH 4.6. After extensive washing, the activity was removed by elution with 50 mM NaAc/0.25 M NaCl/6 M urea, pH 4.6. This fraction was applied to a 500-ml hydroxyapatite column in 80 mM potassium phosphate/6 M urea, pH 6.0, and dissociated from the column in 100 mM potassium phosphate/6 M urea, pH 7.4. The active material was absorbed to a 100-ml heparin-Sepharose column in 50 mM potassium phosphate/0.15 M NaCl/6 M urea, pH 7.4,

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Abbreviation: BMP, bone morphogenetic protein.

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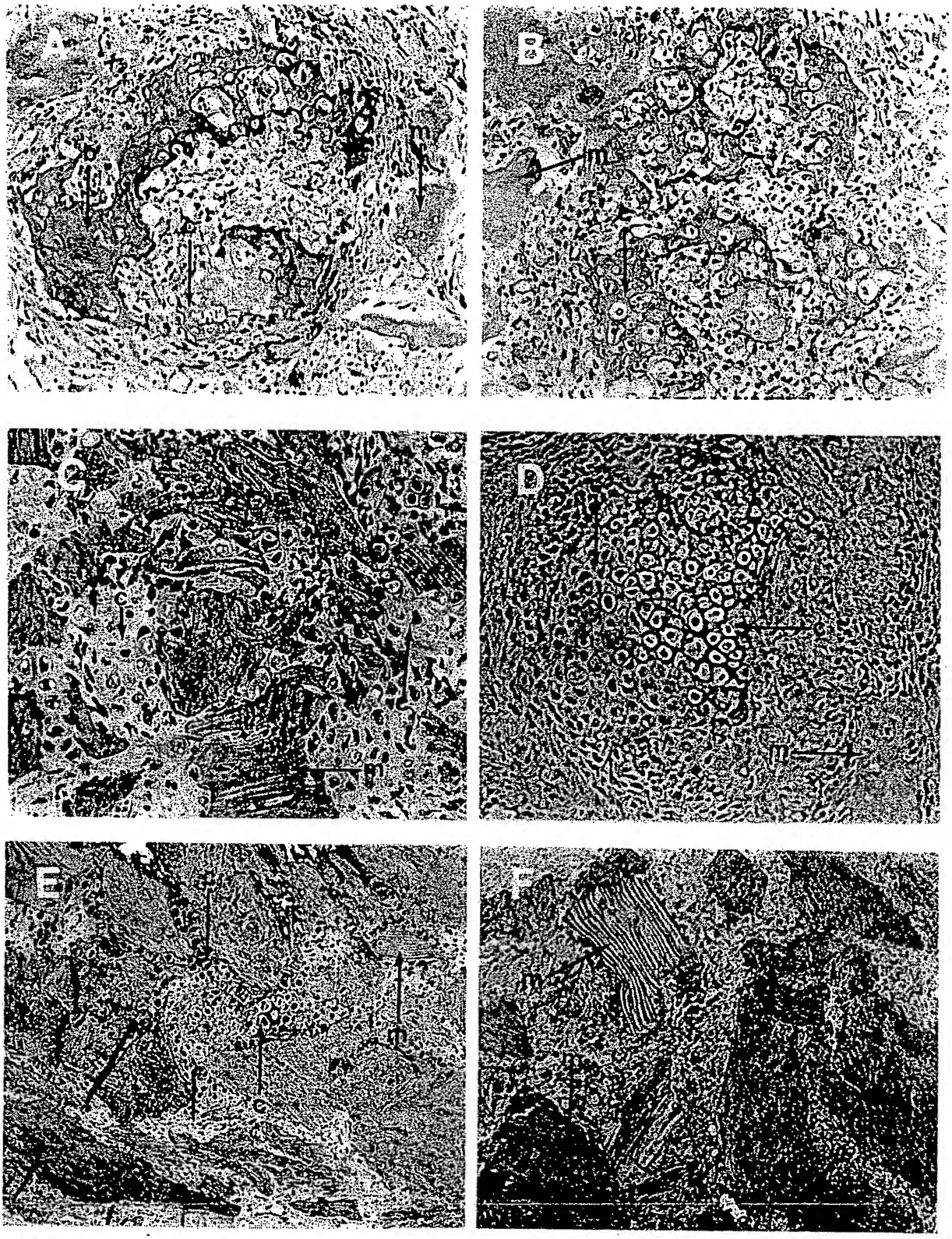

FIG. 1. Cartilage and bone induced by BMP. m, Matrix particles, c, new cartilage, and b, new bone. (*A*) About 10 units of 1000-fold-purified BMP was reconstituted as described; histological scoring of this implant was cartilage (C) +2, bone (B) +3. (See text.) (×65.) (*B*) Activity induced by 3 units of 1000-fold-purified BMP; histological scoring of C+3, B+/−. (×65.) (*C*) Activity induced by 0.2 μg of reverse-phase-purified BMP (1.8 μg of protein); scoring of C+4. (×130.) (*D*) Activity induced by 0.07 μg of BMP as in *C*; scoring of C+2. (×130.) (*E*) Activity induced by 0.02 μg of BMP as in *C*; scoring of C+1. (×65.) (*F*) Implantation of matrix alone subjected to the reconstitution procedure with 0.1% CF_3COOH. (×65.)

Table 1. Summary of purification of BMP activity

Fractionation step	Protein, mg	BMP, units
1 Guanidinium chloride extract	30,000	ND
2 DE-52	13,000	ND
3 CM-cellulose	5,300	660
4 Hydroxyapatite	530	ND
5 Heparin–Sepharose	29	ND
6 Superose	7	200
7 Mono S	≈1	150
8 Reverse phase	≈0.02	120

ND, not determined.

and desorbed with 50 mM potassium phosphate/0.7 M NaCl/6 M urea, pH 7.4. Gel filtration was performed on the heparin-Sepharose-bound protein on Superose 6 and 12 HR 10/30 columns connected in series equilibrated in 4 M guanidinium chloride/20 mM Tris, pH 7.2. Active fractions had a relative migration corresponding to an approximate molecular mass of 30 kDa. Active material was then fractionated on a Mono S column (loading 25 mg of protein per ml of resin) in 50 mM NaoAc/6 M urea, pH 4.6, developed with a gradient from 0 M to 1.0 M NaCl. The active fractions were acidified to pH 3.0 with CF_3COOH and applied to a 0.46 × 25 cm Vydac C_4 column in 0.1% CF_3COOH, and the column was developed with a gradient to 90% acetonitrile/0.1% CF_3COOH.

Analytical methods. Protein was iodinated by the chloramine-T method (15), and molecular mass and isoelectric point analysis were done in the Laemmli system (16) and a Triton X-100/urea system (17). For analysis of BMP activity from these gel systems, samples were heated to 37°C for 15 min in sample buffer. Protein was eluted from the crushed gel slices in 50 mM Tris/0.1% $NaDodSO_4$, pH 7.8. The supernatant was acidified with 10% CF_3COOH to pH 3 and desalted on a 0.46 × 5 cm Vydac C_4 column developed with a gradient of 0.1% CF_3COOH to 90% acetonitrile/0.1% CF_3COOH. The fractions predicted to contain BMP (34–38% acetonitrile) were pooled, and appropriate amounts were assayed as described above. Protein was estimated by the method of Bradford (18) or by absorbance at 280 nm.

Amino acid sequencing. Nonreduced BMP was resolved by preparative $NaDodSO_4$/PAGE, reduced, and alkylated *in situ* by exposure to a combination of 2-mercaptoethanol and 4-vinylpyridine vapors, and then fixed in methanol/acetic acid/water. The fixed gel slice was rinsed in water and neutralized by immersion in a small volume of 0.1 M ammonium bicarbonate solution; finally, the protein was digested from the gel with tosylphenylalanine chloromethyl ketone (TPCK)-treated trypsin (1% by weight). Individual tryptic fragments were isolated after fractionation on a Vydac C_4 column developed in 0.1% CF_3COOH to 0.1% CF_3COOH/95% acetonitrile and sequenced on a model 470A gas-phase sequencer (Applied Biosystems).

RESULTS

Assay. We modified the *in vivo* bone formation assay (5) to increase its sensitivity, shorten its duration, and measure the activity. (*i*) Reconstitution of soluble protein in volatile solvents with inactive bone matrix by lyophilization has proven to be the most reproducible and sensitive procedure for sample preparation—particularly when assaying very low amounts of protein. (*ii*) We determined that the time course for the morphological development in these samples is accelerated compared with that previously described (2, 3, 19), and we were able to remove implants after 7 days instead of the 12–21 days previously required (9, 10). The time-course studies also showed that cartilage formation at 7 days correlated with bone formation at 10–14 days, regardless of whether crude or the most highly purified protein was implanted. (*iii*) We attempted to measure activity by using dose–response studies, where activity was seen within an ≈20-fold range. Each implant was histologically evaluated for the appearance of cartilage and bone, which was scored on a scale of 1–5, as illustrated in Fig. 1. One unit of activity is defined as the minimum amount of BMP required to produce cartilage formation of +2 at an ectopic site 7 days after implantation. Interestingly, we found that increasing dosages accelerate the rate of development in a limited manner, so that at 7 days, 1–2 units of BMP show moderate amount of cartilage (Fig. 1*B*), whereas 5–10 units of the same material show bone as well as cartilage (Fig. 1*A*). Note that the response is the same whether crude or highly purified bovine BMP had been implanted.

Purification. To define a factor that induces the *in vivo* formation of cartilage and bone, we purified BMP from bovine bone, a readily available source. A typical purification starting from 10 kg of bone is summarized in Table 1. Subsequent batches of 10 kg were pooled at intermediate fractionation steps to improve final recovery. Fractionation of BMP revealed several interesting physical properties. Insolubility of the factor or other proteins that copurify with BMP required that all but the last fractionation step be done in the presence of 6 M urea or 4 M guanidinium chloride; however, the activity was extremely stable even under these denaturing conditions. BMP activity binds to heparin-Sepharose, and its complete elution by 0.7 M NaCl shows heparin-binding affinity similar to that of platelet-derived growth factor and much lower than that exhibited by acidic or basic fibroblastic growth factor (20), although the inclusion of urea in the buffers may affect the affinity. BMP has an estimated molecular mass of 30 kDa as determined by gel filtration in guanidinium chloride (Fig. 2*A*). At the next step, Mono S fractionation, two widely separated peaks of BMP with similar activity were sometimes seen (Fig. 2*B*). The physical basis of this heterogeneity was not determined, and only the first peak, containing most of the units, was used for the next step. After the final chromatographic step of reverse-phase HPLC (Fig. 2*C*), the protein had a specific activity of 150 ng of protein per unit, and an overall purification of 300,000-fold relative to the starting guanidinium chloride extract was achieved. The yield of protein, estimated by absorbance at 280 nm, was ≈20 μg per 10 kg of bone; recovery of activity was generally 10–20% based on assay of material purified on CM-cellulose, which removed inhibitory or inflammatory material (5).

The most highly purified BMP was analyzed by PAGE. A nonreducing $NaDodSO_4$ gel of radioiodonated active fractions from the C_4 column (Fig. 2*C*) is shown in Fig. 3*A*; the activity, which peaks in fraction 40, correlates with the 30-kDa protein. As described above, gel filtration experiments also indicated a molecular mass of ≈30 kDa, but the molecular mass of the activity in $NaDodSO_4$/PAGE was needed to confirm the 30-kDa species as the active protein. About 3 μg of protein from the final purification step of a similar BMP preparation was separated on a 15% $NaDodSO_4$ gel, and activity in gel slices was determined as described. As shown in Fig. 3*B*, the activity corresponded to the protein species at 30 kDa, and no activity was seen in any other portion of the gel. Because the 30-kDa protein was approximately one-third of the total protein as determined by silver stain (Fig. 3*B*), the estimate for one unit of BMP activity was revised to ≈50 ng. In a similar experiment, the isoelectric point of BMP was determined to be ≈8.8 in a urea/Triton X-100 electrophoretic system (data not shown).

When the 30-kDa gel-purified BMP was reduced, components of 30, 18, and 16 kDa were seen in $NaDodSO_4$/PAGE analysis (Fig. 4). Based on the radioiodinated material, all three peptides appear equal in quantity. However, because tyrosine content is not known, no conclusions on stoichiometry or on subunit composition can be drawn. More impor-

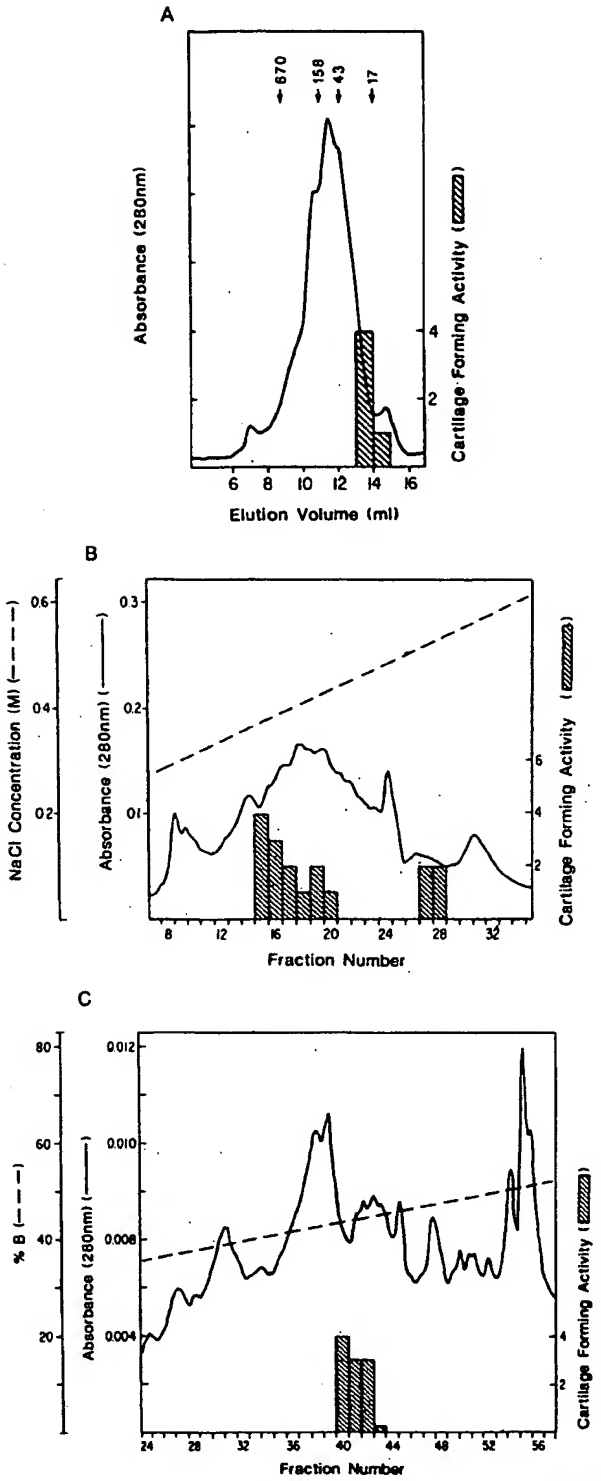

FIG. 2. Chromatography profiles of the last three steps in BMP purification. (*A*) Gel filtration of BMP on Superose 6 and 12 columns connected in series. Elution of Bio-Rad gel filtration markers is shown. BMP emerges at a position corresponding to ≈29 kDa. (*B*) Fraction of BMP on the Mono S column; two peaks of activity are seen. Only the earlier eluting BMP is subsequently purified. (*C*) Fractionation of BMP on a C_4 reverse-phase column. Buffer A is 0.1% CF_3COOH, and buffer B is 0.1% CF_3COOH/90% acetonitrile. Iodination of aliquots from this step is shown in Fig. 3.

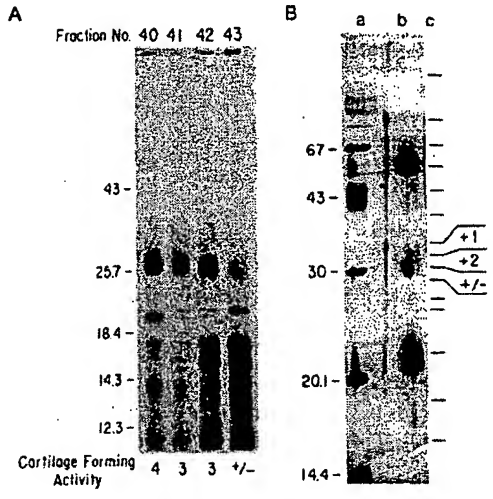

FIG. 3. Molecular mass analysis of BMP. (*A*) $NaDodSO_4$/PAGE analysis of ^{125}I-labeled fractions from the C_4 column fractionation shown in Fig. 2*C*. Fraction number is at top, and activity from 50-μl assays is indicated at bottom. (*B*) Molecular mass determination of BMP. Active reverse-phase purified protein was analyzed on a 15% gel. Gel slices to be analyzed were removed and the gel was silver stained. Lanes: b, 0.1 μg of BMP; c, 3.0 μg of BMP; and a, molecular mass markers in kDa. Gel slices were removed as noted, and activity was determined.

tantly, this analysis indicates that BMP activity might reside in one or more of these peptides.

All BMPs appear to be glycosylated: digestion of components of the 30-kDa BMP gel band with N-Glycanase reduced the observed molecular mass of each component by 3, 2.5, and 2.5 kDa, respectively (Fig. 4). About half of the highly purified BMP activity bound to a Con A–Sepharose column with Triton X-100 and without urea and was removed from the column with α-methyl-D-mannoside (data not shown). These results are consistent with the presence of an α-mannose containing asparagine-linked complex carbohydrate on BMP.

Although the reduced individual peptides of the 30-kDa BMP are easily resolved on $NaDodSO_4$/PAGE, treatment of BMP with reducing agents results in loss of activity (21, data not shown); thus the active component could not be directly analyzed from a reducing $NaDodSO_4$ gel. We attempted to further identify the active BMP species by trying to separate the components of the nonreduced 30-kDa material by

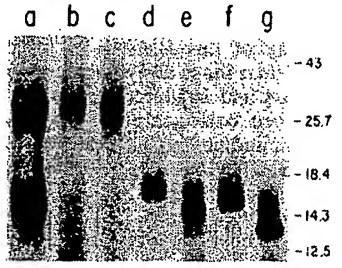

FIG. 4. Subunit composition of BMP. The 30-kDa ^{125}I-labeled BMP was purified from a 15% $NaDodSO_4$ gel and analyzed. Lanes: a, BMP, reduced and alkylated. Components are seen at 30, 18, and 16 kDa, respectively; b, 30-kDa component, reduced and alkylated; c, same 30-kDa component digested with 0.02 unit of N-Glycanase (Genzyme); d, 18-kDa component, reduced and alkylated; e, same 18-kDa component digested with 0.02 unit of N-Glycanase; f, 16-kDa component, reduced and alkylated; and g, same 16-kDa component digested with 0.02 unit of N-Glycanase.

Table 2. Sequence of tryptic fragments from BMP

Fragments	Sequence
BMP mix	
1	A A F L G D I A L D E E D L G
2	A F Q V Q Q A A D L
3	N Y Q D M V V E G
4	F D A Y Y
5	L K P S N ? A T I Q S I V E
16-kDa protein	
6	S L K P S N H A T I Q S ? V
7	S F D A Y Y C S G A
8	V Y P N M T V E S C A
9	V D F A D I ? W

One-letter amino acid code is used.

various methods but were unable to do so without significant loss of protein or activity. Because of the enormous effort required to obtain pure protein and the great complexities involved in characterizing individual proteins, we decided to use amino acid sequence information to obtain cDNA clones for each protein and then characterize each protein on the recombinant level. To ensure the optimum amount of amino acid sequence, the 30-kDa nonreduced-BMP gel band was digested with trypsin, and the tryptic peptides were separated by reverse-phase chromatography. The sequences obtained from individual peptides are shown in Table 2. Additional sequence was obtained from another batch of material that was substantially enriched in the 16-kDa protein and is also included in Table 2.

DISCUSSION

Using a highly specific and sensitive bone-formation assay, we purified a BMP from guanidine extracts of demineralized bone >300,000-fold. As little as 50 ng of the factor can induce the formation of cartilage, although the effective amount of BMP delivered to the site is not known. The activity has been characterized as a basic protein of nonreduced molecular mass of ≈30 kDa, as determined by elution from gels. Gel-purified BMP is composed of three proteins of molecular mass 30, 18, and 16 kDa; because all attempts to separate the components resulted in inactivation, the exact identity of the active material was not determined. The requirements for bone induction in this system may be as simple as the 30-kDa protein alone or as complex as the 30-kDa protein in combination with an 18- and 16-kDa heterodimer.

The high specific activity and the physical properties suggest that the BMP we isolated is a growth factor composed of unusual proteins. Despite differences that might be expected because of the matrix used in the *in vivo* assay system, the specific activity of this BMP is at least an order of magnitude greater than those activities previously reported. The molecular mass and isoelectric point are also distinct from other described osteogenic factors. The osteogenin described by Reddi and colleagues (10) was a protein of 22 kDa, whereas the BMP of Urist *et al.* (9) was an acidic protein of 18.5 kDa; both proteins were derived from bovine bone, and they had *in vivo* activity at 5 μg and 1–5 mg, respectively. Takaoka *et al.* (8) have characterized a 22-kDa bone-inductive factor isolated from a murine osteosarcoma with an unknown specific activity. In addition, a survey of physical properties of other growth factors indicates similarity of BMP only to platelet-derived growth factor; both are basic 30-kDa proteins composed of two disulfide-linked subunits (22). Our experiments with human or porcine platelet-derived growth factor confirm the lack of *in vivo* cartilage-forming activity previously described (10). Also, none of the tryptic peptides isolated from BMP showed any identity to amino acid sequence in the National Biomedical Research Foundation data base.

Because of these results, we were convinced that the criteria of purity, potency, and specificity for another growth factor had been fulfilled by our 30-kDa protein. Because the amount of BMP is so limited, we used sequence information obtained from a mixture of the three proteins to clone the respective genes for further characterization of the individual activities of recombinant factors. Human cDNAs for each of these three proteins have been shown to encode rare proteins (J.M.W., V.R., A. J. Celeste, L. M. Mitsock, M. J. Whitters, R. W. Kriz, R.M.H. and E.A.W., unpublished work). Interestingly, the genes for the 18- and 16-kDa proteins are related, both being members of the inhibin/transforming growth factor type β gene family. More importantly, the recombinant proteins are individually active in cartilage induction, and each of the three factors presumably contributes to the complex process of bone formation.

Examination of BMP activity has been so far restricted to the bone-induction system, and the effect of BMP has been studied only in the very localized space of the implant, whether consisting of protein alone or protein in combination with a matrix. It will be very interesting to discover other *in vitro* and *in vivo* activities and specificities for these factors, and these results should help elucidate the regulation of cartilage and bone growth and differentiation.

We thank J. D'Alessandro, D. McQuaid, C. Senat, R. Palmer, B. Fendrock, R. Kay, and M. Ryan for their assistance and M. Byrne, S. Clark, and E. Fritsch for helpful comments on this manuscript.

1. Urist, M. R. (1965) *Science* 150, 893–899.
2. Reddi, A. H. & Huggins, C. (1972) *Proc. Natl. Acad. Sci. USA* 69, 1601–1605.
3. Firschein, H. E. & Urist, M. R. (1972) *Clin. Orthop. Relat. Res.* 84, 263–275.
4. Urist, M. R., Conover, M. A., Lietze, A., Triffitt, J. T. & DeLange, R. (1981) in *Hormonal Control of Calcium Metabolism*, eds. Cohn, D. V., Talmage, R. V. & Matthews, J. L. (Elsevier North-Holland, Amsterdam), Vol. 511, pp. 307–314.
5. Sampath, T. K. & Reddi, A. H. (1983) *Proc. Natl. Acad. Sci. USA* 80, 6591–6595.
6. Ya Sui, N., Ochi, T., Takaoka, K., Ono, K. & Nakata, Y. (1980) *Biken J.* 23, 83–97.
7. Urist, M. R., Lietze, A. & Dawson, E. (1984) *Clin. Orthop. Relat. Res.* 187, 277–280.
8. Takaoka, K., Yoshikawa, H., Shimizu, N., Ono, K., Amitani, K., Nakata, Y. & Sakamoto, Y. (1981) *Biomed. Res.* 2, 466–471.
9. Urist, M. R., Huo, Y. K., Brownell, A. G., Holh, W. M., Buyske, J., Lietze, A., Tempst, P., Hunkapiller, M. & DeLange, R. J. (1984) *Proc. Natl. Acad. Sci. USA* 81, 371–375.
10. Sampath, T. K., Muthukumaran, N. & Reddi, A. H. (1987) *Proc. Natl. Acad. Sci. USA* 84, 7109–7113.
11. Simpson, E. (1984) *Trends Biochem. Sci.* 9, 527–530.
12. Centrella, M. & Canalis, E. (1985) *Endocr. Rev.* 6, 544–551.
13. Urist, M. R., Iwata, H., Ceccotti, P. L., Dorfman, R. L., Boyd, S. D., McDowell, R. M. & Chien, C. (1973) *Proc. Natl. Acad. Sci. USA* 70, 3511–3515.
14. Urist, M. R., Lietze, A., Mizutani, H., Takagi, K., Triffitt, J. T., Amstutz, J., DeLange, R., Termine, J. & Finerman, G. A. (1982) *Clin. Orthop. Relat. Res.* 162, 219–232.
15. McConahey, P. J. & Dixon, F. J. (1966) *Int. Arch. Allergy Appl. Immunol.* 29, 185–189.
16. Laemmli, U. K. (1970) *Nature (London)* 227, 680–685.
17. Anderson, N. G. & Anderson, N. L. (1978) *Anal. Biochem.* 85, 331–340.
18. Bradford, M. (1976) *Anal. Biochem.* 72, 248–254.
19. Urist, M. R., Jurist, J. M., Dubuc, F. L. & Strates, B. S. (1970) *Clin. Orthop. Relat. Res.* 68, 279–293.
20. Hauschka, P. V., Mavrakos, A. E., Iafrati, M. D., Doleman, S. E. & Klagsbrun, M. (1986) *J. Biol. Chem.* 261, 12665–12674.
21. Urist, M. R., Chang, J. J., Lietze, A., Huo, Y. K., Brownell, A. G. & DeLange, R. J. (1987) *Methods Enzymol.* 146, 294–312.
22. Ross, R., Raines, E. W. & Bowen-Pope, D. F. (1984) *Cell* 46, 155–169.

DANIEL J. CAPON, ARTHUR WEISS, BRIAN A. IRVING, MARGO R.ROBERTS, and KRISZTINA ZSEBO, Appellants, v. ZELIG ESHHAR, DANIEL SCHINDLER,TOVA WAKS, and GIDEON GROSS, Cross-Appellants, v. JON DUDAS, Director of thePatent and Trademark Office, Intervenor.
03-1480, 03-1481

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

418 F.3d 1349; 2005 U.S. App. LEXIS 16865; 76 U.S.P.Q. (BNA)1078

August 12, 2005, Decided

PRIOR HISTORY: [**1] Appealed from: United States Patent and Trademark Office Board of Patent Appeals and Interferences. (Interference No. 103,887)

CASE SUMMARY:

PROCEDURAL POSTURE: Both parties to a patent interference proceeding between United States Patent No. 6,407,221 ('221 patent) and patent application Serial No. 08/084,994 ('994 application) appealed the decision of the Board of Patent Appeals and Interferences of the United States Patent and Trademark Office that the specification of neither party met the written description requirement of the patent statute, dissolving the interference and cancelling the claims.

OVERVIEW: The patent and application were directed to the production of chimeric genes designed to enhance immune responses. The Board held that neither party's specification provided the requisite description of the full scope of the chimeric DNA or encoded proteins. The Board Director argued that it could not be known whether all of the permutations and combinations covered by the claims would be effective for the intended purpose, and that the claims were too broad because they might include inoperative species. Both parties presented specific examples of the production of specified chimeric genes. The court held it was not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention. The court held that the Board erred in ruling that 35 U.S.C.S. § 112 imposed a per se rule requiring recitation in the specification of the nucleotide sequence of claimed DNA, when that sequence was already known in the field.

OUTCOME: The Board's decision was vacated and the case remanded to the Board for further proceedings.

CORE TERMS: gene, chimeric, protein, cell, segment, domain, invention, chain, specification, antibody, sequence, patent, written description, encode, encoding, extracellular, nucleotide, receptor, comprising, inventor, single-chain, cytoplasmic, disclosure, immune system, endogenous, membrane, ligand, specificity, generic, novel

LexisNexis(R) Headnotes

Administrative Law: Judicial Review: Standards of Review: Arbitrary & Capricious Review
[HN1] In accordance with the Administrative Procedure Act, the law as interpreted and applied by an agency receives plenary review on appeal, and the agency's factual findings are reviewed to determine whether they were arbitrary, capricious, or unsupported by substantial evidence in the administrative record.

Patent Law: Claims & Specifications: Definiteness: Precision Standards
[HN2] The required content of a patent specification is set forth in 35 U.S.C.S. § 112. The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Patent Law: Claims & Specifications: Definiteness: Precision Standards
[HN3] The "written description" requirement of 35 U.S.C.S. § 112 implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed.

Patent Law: Claims & Specifications: Description Requirement: General Overview

[HN4] It is well recognized that in the "unpredictable" fields of science, it is appropriate to recognize the variability in the science in determining the scope of the coverage to which an inventor is entitled. Such a decision usually focuses on the exemplification in the specification.

Patent Law: Claims & Specifications: Description Requirement: Proof
[HN5] The determination of what is needed to support generic patent claims to biological subject matter depends on a variety of factors, such as the existing knowledge in the particular field, the extent and content of the prior art, the maturity of the science or technology, the predictability of the aspect at issue, and other considerations appropriate to the subject matter.

Patent Law: Claims & Specifications: Description Requirement: Proof
[HN6] It is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention.

Patent Law: Claims & Specifications: Description Requirement: Written Description Versus Enablement
[HN7] Although the legal criteria of enablement and written description are related and are often met by the same disclosure, they serve discrete legal requirements.

Patent Law: Claims & Specifications: Description Requirement: Proof
[HN8] The predictability or unpredictability of the science is relevant to deciding how much experimental support is required to adequately describe the scope of an invention.

COUNSEL: Steven B. Kelber, Piper Rudnick, LLP, of Washington, DC, argued for appellants.

Roger L. Browdy, Browdy and Neimark, P.L.L.C., of Washington, DC, argued for cross-appellants.

Mary L. Kelly, Associate Solicitor, Office of the Solicitor, United States Patent and Trademark Office, of Arlington, Virginia, argued for intervenor. With her on the brief were John M. Whealan, Solicitor and Stephen Walsh, Associate Solicitor.

JUDGES: Before NEWMAN, MAYER, * and GAJARSA, Circuit Judges.

* Haldane Robert Mayer vacated the position of Chief Judge on December 24, 2004.

OPINIONBY: NEWMAN

OPINION:
[*1350] NEWMAN, Circuit Judge.

Both of the parties to a patent interference proceeding have appealed the decision of the Board of Patent Appeals and Interferences of the United States Patent and Trademark Office, wherein the Board held that the specification of neither party met the written description requirement of the patent statute. Capon v. Eshhar, Interf. No. 103,887 (Bd. Pat. App. & Interf. Mar. 26, 2003). The Board dissolved the interference and cancelled all[**2] of the claims of both parties corresponding to the interference count. With this ruling, the Board terminated the proceeding and did not reach the question of priority of invention. We conclude that the Board erred in its application of the law of written description. The decision is vacated and the case is remanded to the Board for further proceedings.

BACKGROUND

Daniel J. Capon, Arthur Weiss, Brian A. Irving, Margo R. Roberts, and Krisztina Zsebo (collectively "Capon") and Zelig Eshhar, Daniel Schindler, Tova Waks, and [*1351] Gideon Gross (collectively "Eshhar") were the parties to an interference proceeding between Capon's *United States Patent No. 6,407,221* ("the *'221 patent*") entitled "Chimeric Chains for Receptor-Associated Signal Transduction Pathways" and Eshhar's patent application Serial No. 08/084,994 ("the '994 application") entitled "Chimeric Receptor Genes and Cells Transformed Therewith." Capon's *Patent No. 5,359,046* ("the '046 patent"), parent of the *'221 patent*, was also included in the interference but was held expired for non-payment of a maintenance fee. The PTO included the *'046* patent in its decision and in its argument of this appeal. n1

----------Footnotes----------

n1 Although Capon is designated as appellant and Eshhar as cross-appellant, both appealed the Board's decision. See *Fed. R. App. P. 28(h)*. The Director of the PTO intervened to support the Board, and has fully participated in this appeal.

----------End Footnotes----------

[**3]

A patent interference is an administrative proceeding pursuant to *35 U.S.C. §§ 102(g)* and *135(a)*, conducted for the purpose of determining which of competing applicants is the first inventor of common subject matter. An interference is instituted after the separate patent applications have been examined and found to contain patentable subject matter. Capon's patents had been examined and had issued before this interference was instituted, and Eshhar's application had been examined and allowed but a patent had not yet issued.

During an interference proceeding the Board is authorized to determine not only priority of invention but also to redetermine patentability. *35 U.S.C. § 6(b)*. The question of patentability of the claims of both parties was raised sua sponte by an administrative patent judge during the preliminary proceedings. Thereafter the Board conducted an inter partes proceeding limited to this question, receiving evidence and argument. The Board then invalidated all of the claims that had been designated as corresponding to the count of the interference, viz., all of the claims of the Capon *'221 patent*, claims 5-8 of [**4]the Capon *'046* patent, and claims 1-7, 9-20, and 23 of the Eshhar '994 application.

[HN1] In accordance with the Administrative Procedure Act, the law as interpreted and applied by the agency receives plenary review on appeal, and the agency's factual findings are reviewed to determine whether they were arbitrary, capricious, or unsupported by substantial evidence in the administrative record. See *5 U.S.C. § 706(2)*; *Dickinson v. Zurko, 527 U.S. 150, 164-65, 144 L. Ed. 2d 143, 119 S. Ct. 1816 (1999)*; *In re Gartside, 203 F.3d 1305, 1315 (Fed. Cir. 2000)*.

The Invention

A chimeric gene is an artificial gene that combines segments of DNA in a way that does not occur in nature. The *'221 patent* and '994 application are directed to the production of chimeric genes designed to enhance the immune response by providing cells with specific cell-surface antibodies in a form that can penetrate diseased sites, such as solid tumors, that were not previously reachable. The parties explain that their invention is a way of endowing immune cells with antibody-type specificity, by combining known antigen-binding-domain producing DNA and known lymphocyte-receptor-protein[**5] producing DNA into a unitary gene that can express a unitary polypeptide chain. Eshhar summarized the problem to which the invention is directed:

Antigen-specific effector lymphocytes, such as tumor-specific T cells, are very rare, individual-specific, limited in their recognition spectrum and difficult to obtain against most malignancies. Antibodies, on the other hand, are readily [*1352] obtainable, more easily derived, have wider spectrum and are not individual-specific. The major problem of applying specific antibodies for cancer immunotherapy lies in the inability of sufficient amounts of monoclonal antibodies (mAb) to reach large areas within solid tumors.

Technical Paper Explaining Eshhar's Invention, at 6.

The inventions of Capon and Eshhar are the chimeric DNA that encodes single-chain chimeric proteins for expression on the surface of cells of the immune system, plus expression vectors and cells transformed by the chimeric DNA. The experts for both parties explain that the invention combines selected DNA segments that are both endogenous and nonendogenous to a cell of the immune system, whereby the nonendogenous segment encodes the single-chain variable ("scFv") [**6] domain of an antibody, and the endogenous segment encodes cytoplasmic, transmembrane, and extracellular domains of a lymphocyte signaling protein. They explain that the scFv domain combines the heavy and light variable ("Fv") domains of a natural antibody, and thus has the same specificity as a natural antibody. Linking this single chain domain to a lymphocyte signaling protein creates a chimeric scFv-receptor ("scFvR") gene which, upon transfection into a cell of the immune system, combines the specificity of an antibody with the tissue penetration, cytokine production, and target-cell destruction capability of a lymphocyte.

The parties point to the therapeutic potential if tumors can be infiltrated with specifically designed immune cells of appropriate anti-tumor specificity.

The Eshhar Claims

The Board held unpatentable the following claims of Eshhar's '994 application; these were all of the '994 claims that had been designated as corresponding to the count of the interference. Eshhar's claim 1 was the designated count.

1. A chimeric gene comprising

a first gene segment encoding a single-chain Fv domain (scFv) of a specific antibody and

a second gene segment[**7] encoding partially or entirely the transmembrane and cytoplasmic, and optionally the extracellular, domains of an endogenous protein

wherein said endogenous protein is expressed on the surface of cells of the immune system and triggers activation and/or proliferation of said cells,

which chimeric gene, upon transfection to said cells of the immune system, expresses said scFv domain and said domains of said endogenous protein in one single chain on the surface of the transfected cells such that the transfected cells are triggered to activate and/or proliferate and have MHC nonrestricted antibody-type specificity when said expressed scFV domain binds to its antigen.

2. A chimeric gene according to claim 1 wherein the second gene segment further comprises partially or entirely the extracellular domain of said endogenous protein.

3. A chimeric gene according to claim 1 wherein the first gene segment encodes the scFv domain of an antibody against tumor cells.

4. A chimeric gene according to claim 1 wherein the first gene segment encodes the scFv domain of an antibody against virus infected cells.

5. A chimeric gene according to claim 4 wherein the virus is HIV.

6. [**8] A chimeric gene according to claim 1 wherein the second gene segment encodes a lymphocyte receptor chain.

[*1353] 7. A chimeric gene according to claim 6 wherein the second gene segment encodes a chain of the T cell receptor.

9. A chimeric gene according to claim 7 wherein the second gene segment encodes the a, B, y, or o chain of the antigen-specific T cell receptor.

10. A chimeric gene according to claim 1 wherein the second gene segment encodes a polypeptide of the TCR/CD3 complex.

11. A chimeric gene according to claim 10 wherein the second gene segment encodes the zeta or eta isoform chain.

12. A chimeric gene according to claim 1 wherein the second gene segment encodes a subunit of the Fc receptor or IL-2 receptor.

13. A chimeric gene according to claim 12 wherein the second gene segment encodes a common subunit of IgE and IgG binding Fc receptors.

14. A chimeric gene according to claim 13 wherein said subunit is the gamma subunit.

15. A chimeric gene according to claim 13 wherein the second gene segment encodes the CD16a chain of the FcyRIII or FcyRII.

16. A chimeric gene according to claim 12 wherein the second gene segment encodes the a[**9] or B subunit of the IL-2 receptor.

17. An expression vector comprising a chimeric gene according to claim 1.

18. A cell of the immune system endowed with antibody specificity transformed with an expression vector according to claim 17.

19. A cell of the immune system endowed with antibody specificity comprising a chimeric gene according to claim 1.

20. A cell if the immune system according to claim 19 selected from the group consisting of a natural killer cell, a lymphokine activated killer cell, a cytotoxic T cell, a helper T cell and a subtype thereof.

23. A chimeric gene according to claim 1 wherein said endogenous protein is a lymphocyte receptor chain, a polypeptide of the TCR/CD3 complex, or a subunit of the Fc or IL-2 receptor.

The Board did not discuss the claims separately, and held that the specification failed to satisfy the written description requirement as to all of these claims.

The Capon Claims

Claims 1-10, all of the claims of the *'221 patent*, were held unpatentable on written description grounds. Claims 1-6 are directed to the chimeric DNA, claims 7, 8, and 10 to the corresponding cell comprising the DNA, and claim 9 to [**10]the chimeric protein:

1. A chimeric DNA encoding a membrane bound protein, said chimeric DNA comprising in reading frame:

DNA encoding a signal sequence which directs said membrane bound protein to the surface membrane;

DNA encoding a non-MHC restricted extracellular binding domain which is obtained from a single chain antibody that binds specifically to at least one ligand, wherein said at least one ligand is a protein on the surface of a cell or a viral protein;

DNA encoding a transmembrane domain which is obtained from a protein selected from the group consisting of CD4, CD8, immunoglobulin, the CD3 zeta chain, the CD3 gamma chain, the CD3 delta chain and the CD3 epsilon chain; and

DNA encoding a cytoplasmic signal-transducing domain of a protein that activates an intracellular messenger system which is obtained from CD3 zeta,

[*1354] wherein said extracellular domain and said cytoplasmic domain are not naturally joined together, and said cytoplasmic domain is not naturally joined to an extracellular ligand-binding domain, and when said chimeric DNA is expressed as a membrane bound protein in a host cell under conditions suitable for expression, said membrane bound protein[**11] initiates signaling in said host cell when said extracellular domain binds said at least one ligand.

2. The DNA of claim 1, wherein said single-chain antibody recognizes an antigen selected from the group consisting of viral antigens and tumor cell associated antigens.

3. The DNA of claim 2 wherein said single-chain antibody is specific for the HIV env glycoprotein.

4. The DNA of claim 1, wherein said transmembrane domain is naturally joined to said cytoplasmic domain.

5. An expression cassette comprising a transcriptional initiation region, the DNA of claim 1 under the transcriptional control of said transcriptional initiation region, and a transcriptional termination region.

6. A retroviral RNA or DNA construct comprising the expression cassette of claim 5.

7. A cell comprising the DNA of claim 1.

8. The cell of claim 7, wherein said cell is a human cell.

9. A chimeric protein comprising in the N-terminal to C-terminal direction:

a non-MHC restricted extracellular binding domain which is obtained from a single chain antibody that binds specifically to at least one ligand, wherein said at least one ligand is a protein on the surface of a cell[**12] or a viral protein;

a transmembrane domain which is obtained from a protein selected from the group consisting CD4, CD8, immunoglobulin, the CD3 zeta chain, the CD3 gamma chain, the CD3 delta chain and the CD3 epsilon chain; and

a cytoplasmic signal-transducing domain of a protein that activates an intracellular messenger system which is obtained from CD3 zeta,

wherein said extracellular domain and said cytoplasmic domain are not naturally joined together, and said cytoplasmic domain is not naturally joined to an extracellular ligand-binding domain, and when said chimeric protein is expressed as a membrane bound protein in a host cell under conditions suitable for expression, said membrane bound protein initiates signaling in said host cell when said extracellular domain binds said at least one ligand.

10. A mammalian cell comprising as a surface membrane protein, the protein of claim 9.

In addition, claims 5, 6, 7, and 8 of Capon's *'046* patent were held unpatentable. These claims are directed to chimeric DNA sequences where the encoded extracellular domain is a single-chain antibody containing ligand binding activity.

The Board Decision

The Board presumed[**13] enablement by the specifications of the *'221 patent* and '994 application of the full scope of their claims, and based its decision solely on the ground of failure of written description. The Board held that neither party's specification provides the requisite description of the full scope of the chimeric DNA or encoded proteins, by reference to knowledge in the art of the "structure, formula, chemical name, or physical properties" of the DNA or the proteins. In the Board's words:

[*1355] We are led by controlling precedent to understand that the full scope of novel chimeric DNA the parties claim is not described in their specifications under *35 U.S.C. § 112*, first paragraph, by reference to contemporary and/or prior knowledge in the art of the structure, formula, chemical name, or physical properties of many protein domains, and/or DNA sequences which

encode many protein domains, which comprise single-chain proteins and/or DNA constructs made in accordance with the plans, schemes, and examples thereof the parties disclose.

Bd. op. at 4. As controlling precedent the Board cited *Regents of the University of California v. Eli Lilly & Co., 119 F.3d 1559 (Fed. Cir. 1997)*;[**14] *Fiers v. Revel, 984 F.2d 1164 (Fed. Cir. 1993)*; *Amgen, Inc. v. Chugai Pharmaceutical Co., 927 F.2d 1200 (Fed. Cir. 1991)*; and *Enzo Biochem, Inc. v. Gen-Probe, Inc., 296 F.3d 1316 (Fed. Cir. 2002)*. The Board summarized its holding as follows:

Here, both Eshhar and Capon claim novel genetic material described in terms of the functional characteristics of the protein it encodes. Their specifications do not satisfy the written description requirement because persons having ordinary skill in the art would not have been able to visualize and recognize the identity of the claimed genetic material without considering additional knowledge in the art, performing additional experimentation, and testing to confirm results.

Bd. op. at 89.

DISCUSSION

Eshhar and Capon challenge both the Board's interpretation of precedent and the Board's ruling that their descriptions are inadequate. Both parties explain that their chimeric genes are produced by selecting and combining known heavy-and light-chain immune-related DNA segments, using known DNA-linking procedures. The specifications of both parties describe procedures for identifying[**15] and obtaining the desired immune-related DNA segments and linking them into the desired chimeric genes. Both parties point to their specific examples of chimeric DNA prepared using identified known procedures, along with citation to the scientific literature as to every step of the preparative method.

The parties presented expert witnesses who placed the invention in the context of prior knowledge and explained how the descriptive text would be understood by persons of skill in the field of the invention. The witnesses explained that the principle of forming chimeric genes from selected segments of DNA was known, as well as their methods of identifying, selecting, and combining the desired segments of DNA. Dr. Eshhar presented an expert statement wherein he explained that the prior art contains extensive knowledge of the nucleotide structure of the various immune-related segments of DNA; he stated that over 785 mouse antibody DNA light chains and 1,327 mouse antibody DNA heavy chains were known and published as early as 1991. Similarly Capon's expert Dr. Desiderio discussed the prior art, also citing scientific literature:

The linker sequences disclosed in the *'221 patent*[**16] (col. 24, lines 4 and 43) used to artificially join a heavy and light chain nucleic acid sequence and permit functional association of the two ligand binding regions were published by 1990, as were the methods for obtaining the mature sequences of the desired heavy and light chains for constructing a SAb (Exhibit 47, Batra et al., J., Biol. Chem., 1990; Exhibit 48, Bird et al., Science, 1988; Exhibit 50, Huston et al., PNAS, [*1356] 1988; Exhibit 51, Chaudhary, PNAS, 1990, Exhibit 56, Morrison et al., Science, 1985; Exhibit 53, Sharon et al., Nature 1984).

Desiderio declaration at 4 P11.

Both parties stated that persons experienced in this field would readily know the structure of a chimeric gene made of a first segment of DNA encoding the single-chain variable region of an antibody, and a second segment of DNA encoding an endogenous protein. They testified that re-analysis to confirm these structures would not be needed in order to know the DNA structure of the chimeric gene, and that the Board's requirement that the specification must reproduce the "structure, formula, chemical name, or physical properties" of these DNA combinations had been overtaken by the state of the science. [**17]They stated that where the structure and properties of the DNA components were known, reanalysis was not required.

Eshhar's specification contains the nucleotide sequences of sixteen different receptor primers and four different scFv primers from which chimeric genes encoding scFvR may be obtained, while Capon's specification cites literature sources of such information. Eshhar's specification shows the production of chimeric genes encoding scFvR using primers, as listed in Eshhar's Table I. Capon stated that natural genes are isolated and joined using conventional methods, such as the polymerase chain reaction or cloning by primer repair. Capon, like Eshhar, discussed various known procedures for identifying, obtaining, and linking DNA segments, accompanied by experimental examples. The Board did not dispute that persons in this field of science could determine the structure or formula of the linked DNA from the known structure or formula of the components.

The Board stated that "controlling precedent" required inclusion in the specification of the complete nucleotide sequence of "at least one" chimeric gene. Bd. op. at 4. The Board also objected that the claims were broader than

[**18]the specific examples. Eshhar and Capon each responds by pointing to the scientific completeness and depth of their descriptive texts, as well as to their illustrative examples. The Board did not relate any of the claims, broad or narrow, to the examples, but invalidated all of the claims without analysis of their scope and the relation of claim scope to the details of the specifications.

Eshhar and Capon both argue that they have set forth an invention whose scope is fully and fairly described, for the nucleotide sequences of the DNA in chimeric combination is readily understood to contain the nucleotide sequences of the DNA components. Eshhar points to the general and specific description in his specification of known immune-related DNA segments, including the examples of their linking. Capon points similarly to his description of selecting DNA segments that are known to express immune-related proteins, and stresses the existing knowledge of these segments and their nucleotide sequences, as well as the known procedures for selecting and combining DNA segments, as cited in the specification.

Both parties argue that the Board misconstrued precedent, and that precedent does not establish[**19] a per se rule requiring nucleotide-by-nucleotide re-analysis when the structure of the component DNA segments is already known, or readily determined by known procedures.

The Statutory Requirement

[HN2] The required content of the patent specification is set forth in *Section 112 of Title 35*:

§ 112 P1. The specification shall contain a written description of the invention, [*1357] and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

[HN3] The "written description" requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed. See *Enzo Biochem, 296 F.3d at 1330* (the written description requirement "is the quid pro quo[**20] of the patent system; the public must receive meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time"); *Reiffin v. Microsoft Corp., 214 F.3d 1342, 1345-46 (Fed. Cir. 2000)* (the purpose of the written description requirement "is to ensure that the scope of the right to exclude ...does not overreach the scope of the inventor's contribution to the field of art as described in the patent specification"); *In re Barker, 559 F.2d 588, 592 n. 4 (CCPA 1977)* (the goal of the written description requirement is "to clearly convey the information that an applicant has invented the subject matter which is claimed"). The written description requirement thus satisfies the policy premises of the law, whereby the inventor's technical/scientific advance is added to the body of knowledge, as consideration for the grant of patent exclusivity.

The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented[**21] advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

For the chimeric genes of the Capon and Eshhar inventions, the law must take cognizance of the scientific facts. The Board erred in refusing to consider the state of the scientific knowledge, as explained by both parties, and in declining to consider the separate scope of each of the claims. None of the cases to which the Board attributes the requirement of total DNA re-analysis, i.e., Regents v. Lilly, Fiers v. Revel, Amgen, or Enzo Biochem, require a re-description of what was already known. In *Lilly, 119 F.3d at 1567*, the cDNA for human insulin had never been characterized. Similarly in *Fiers, 984 F.2d at 1171*, much of the DNA sought to be claimed was of unknown structure, whereby this court viewed the breadth of the claims as embracing a "wish" or research "plan." In *Amgen, 927 F.2d at 1206*, the court explained that a novel gene was [**22]not adequately characterized by its biological function alone because such a description would represent a mere "wish to know the identity" of the novel material. In *Enzo Biochem, 296 F.3d at 1326*, this court reaffirmed that deposit of a physical sample may replace words when description is beyond present scientific capability. In *Amgen Inc. v. Hoechst Marion Roussel, Inc., 314 F.3d 1313, 1332 (Fed. Cir. 2003)* the court explained further that the written description requirement may be satisfied "if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure." These evolving principles were applied in *Noelle [*1358] v. Lederman, 355 F.3d 1343, 1349 (Fed. Cir. 2004)*, where the court affirmed that the human antibody there at issue was not adequately

described by the structure and function of the mouse antigen; and in *University of Rochester v. G.D. Searle & Co., 358 F.3d 916, 925-26 (Fed. Cir. 2004)*, where the court affirmed that the description of the COX-2 enzyme did not serve to describe unknown compounds capable of selectively inhibiting the enzyme.

The "written description"[**23] requirement must be applied in the context of the particular invention and the state of the knowledge. The Board's rule that the nucleotide sequences of the chimeric genes must be fully presented, although the nucleotide sequences of the component DNA are known, is an inappropriate generalization. When the prior art includes the nucleotide information, precedent does not set a per se rule that the information must be determined afresh. Both parties state that a person experienced in the field of this invention would know that these known DNA segments would retain their DNA sequences when linked by known methods. Both parties explain that their invention is not in discovering which DNA segments are related to the immune response, for that is in the prior art, but in the novel combination of the DNA segments to achieve a novel result.

The "written description" requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution. Both Eshhar and Capon explain that this invention does not concern[**24] the discovery of gene function or structure, as in Lilly. The chimeric genes here at issue are prepared from known DNA sequences of known function. The Board's requirement that these sequences must be analyzed and reported in the specification does not add descriptive substance. The Board erred in holding that the specifications do not meet the written description requirement because they do not reiterate the structure or formula or chemical name for the nucleotide sequences of the claimed chimeric genes.

Claim Scope

There remains the question of whether the specifications adequately support the breadth of all of the claims that are presented. The Director argues that it cannot be known whether all of the permutations and combinations covered by the claims will be effective for the intended purpose, and that the claims are too broad because they may include inoperative species. The inventors say that they have provided an adequate description and exemplification of their invention as would be understood by persons in the field of the invention. They state that biological properties typically vary, and that their specifications provide for evaluation of the effectiveness[**25] of their chimeric combinations.

[HN4] It is well recognized that in the "unpredictable" fields of science, it is appropriate to recognize the variability in the science in determining the scope of the coverage to which the inventor is entitled. Such a decision usually focuses on the exemplification in the specification. See, e.g., *Enzo Biochem, 296 F.3d at 1327-28* (remanding for district court to determine "whether the disclosure provided by the three deposits in this case, coupled with the skill of the art, describes the genera of claims 1-3 and 5"); *Lilly, 119 F.3d at 1569* (genus not described where "a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus" had not been provided); *In re Gosteli, 872 F.2d 1008, 1012 (Fed. Cir. 1989)* (two chemical compounds were insufficient [*1359] description of subgenus); *In re Smith, 59 C.C.P.A. 1025, 458 F.2d 1389, 1394-95 (CCPA 1972)* (disclosure of genus and one species was not sufficient description of intermediate subgenus); *In re Grimme, 47 C.C.P.A. 785, 274 F.2d 949, 952, 1960 Dec. Comm'r Pat. 123 (CCPA 1960)* (disclosure of single example and[**26] statement of scope sufficient disclosure of subgenus).

Precedent illustrates that [HN5] the determination of what is needed to support generic claims to biological subject matter depends on a variety of factors, such as the existing knowledge in the particular field, the extent and content of the prior art, the maturity of the science or technology, the predictability of the aspect at issue, and other considerations appropriate to the subject matter. See, e.g., *In re Wallach, 378 F.3d 1330, 1333-34 (Fed. Cir. 2004)* (an amino acid sequence supports "the entire genus of DNA sequences" that can encode the amino acid sequence because "the state of the art has developed" such that it is a routine matter to convert one to the other); *University of Rochester, 358 F.3d at 925* (considering whether the patent disclosed the compounds necessary to practice the claimed method, given the state of technology); *Singh v. Brake, 317 F.3d 1334, 1343, 48 Fed. Appx. 766 (Fed. Cir. 2002)* (affirming adequacy of disclosure by distinguishing precedent in which the selection of a particular species within the claimed genus had involved "highly unpredictable results").

[HN6] It[**27] is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention. See *In re Angstadt, 537 F.2d 498, 504 (CCPA 1976)* ("The examples, both operative and inoperative, are the best guidance this art permits, as far as we can conclude from the record"). While the Board is correct that a generic invention requires adequate support, the sufficiency of the support must be determined in the

particular case. Both Eshhar and Capon present not only general teachings of how to select and recombine the DNA, but also specific examples of the production of specified chimeric genes. For example, Eshhar points out that in Example 1 of his specification the FcR . chain was used, which chain was amplified from a human cDNA clone, using the procedure of Kuster, H. et al., J. Biol. Chem., 265:6448-6451 (1990), which is cited in the specification and reports the complete sequence of the FcRy chain. Eshhar's Example 1 also explains the source of the genes that provide the heavy and light chains of the single chain antibody,[**28] citing the PhD thesis of Gideon Gross, a co-inventor, which cites a reference providing the complete sequence of the Sp6 light chain gene used to construct the single-chain antibody. Eshhar states that the structure of the Sp6 heavy chain antibody was well known to those of skill in the art and readily accessible on the internet in a database as entry EMBL: MMSP6718. Example 5 at page 54 of the Eshhar specification cites Ravetch et al., J. Exp. Med., 170:481-497 (1989) for the method of producing the CD16 a DNA clone that was PCR amplified; this reference published the complete DNA sequence of the CD16 a chain, as discussed in paragraph 43 of the Eshhar Declaration. Example 3 of the Eshhar specification uses the DNA of the monoclonal anti-HER2 antibody and states that the N29 hybridoma that produces this antibody was deposited with the Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris, on August 19, 1992, under Deposit No. CNCM I-1262. It is incorrect to criticize the methods, examples, and referenced prior art of the Eshhar specification as but "a few PCR primers and probes," as does the Director's brief.

[*1360] Capon's Example 3 provides a detailed description[**29] of the creation and expression of single chain antibody fused with T-cell receptor zeta chain, referring to published vectors and procedures. Capon, like Eshhar, describes gene segments and their ligation to form chimeric genes. Although Capon includes fewer specific examples in his specification than does Eshhar, both parties used standard systems of description and identification, as well as known procedures for selecting, isolating, and linking known DNA segments. Indeed, the Board's repeated observation that the full scope of all of the claims appears to be "enabled" cannot be reconciled with the Board's objection that only a "general plan" to combine unidentified DNA is presented. See *In re Wands, 858 F.2d 731, 736-37 (Fed. Cir. 1988)* (experimentation to practice invention must not be "undue" for invention to be considered enabled).

The PTO points out that for biochemical processes relating to gene modification, protein expression, and immune response, success is not assured. However, generic inventions are not thereby invalid. Precedent distinguishes among generic inventions that are adequately supported, those that are merely a "wish" or "plan," the words of[**30] *Fiers v. Revel, 984 F.2d at 1171*, and those in between, as illustrated by *Noelle v. Lederman, 355 F.3d at 1350*; the facts of the specific case must be evaluated. The Board did not discuss the generic concept that both Capon and Eshhar described -- the concept of selecting and combining a gene sequence encoding the variable domain of an antibody and a sequence encoding a lymphocyte activation protein, into a single DNA sequence which, upon expression, allows for immune responses that do not occur in nature. The record does not show this concept to be in the prior art, and includes experimental verification as well as potential variability in the concept.

Whether the inventors demonstrated sufficient generality to support the scope of some or all of their claims, must be determined claim by claim. The Board did not discuss the evidence with respect to the generality of the invention and the significance of the specific examples, instead simply rejecting all the claims for lack of a complete chimeric DNA sequence. As we have discussed, that reasoning is inapt for this case. The Board's position that the patents at issue were merely an "invitation to[**31] experiment" did not distinguish among the parties' broad and narrow claims, and further concerns enablement more than written description. See *Adang v. Fischhoff, 286 F.3d 1346, 1355 (Fed. Cir. 2002)* (enablement involves assessment of whether one of skill in the art could make and use the invention without undue experimentation); *In re Wright, 999 F.2d 1557, 1561 (Fed. Cir. 1993)* (same). [HN7] Although the legal criteria of enablement and written description are related and are often met by the same disclosure, they serve discrete legal requirements.

[HN8] The predictability or unpredictability of the science is relevant to deciding how much experimental support is required to adequately describe the scope of an invention. Our predecessor court summarized in *In re Storrs, 44 C.C.P.A. 981, 245 F.2d 474, 478, 1957 Dec. Comm'r Pat. 361 (CCPA 1957)* that "it must be borne in mind that, while it is necessary that an applicant for a patent give to the public a complete and adequate disclosure in return for the patent grant, the certainty required of the disclosure is not greater than that which is reasonable, having due regard to the subject matter involved." This aspect may[**32] warrant exploration on remand.

In summary, the Board erred in ruling that *§ 112* imposes a per se rule requiring recitation in the specification of the nucleotide [*1361] sequence of claimed DNA, when that sequence is already known in the field. However, the Board did not explore the support

for each of the claims of both parties, in view of the specific examples and general teachings in the specifications and the known science, with application of precedent guiding review of the scope of claims.

We remand for appropriate further proceedings.

VACATED AND REMANDED

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PERSPECTIVES IN BASIC SCIENCE

The family of bone morphogenetic proteins

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The family of bone morphogenetic proteins. Bone morphogenetic proteins (BMPs) are secreted signaling molecules belonging to the transforming growth factor-β (TGF-β) superfamily of growth factors. The first BMPs were originally identified by their ability to induce ectopic bone formation when implanted under the skin of rodents. In this ectopic overexpression assay, there was a recapitulation of all the events occurring during skeletogenesis. This latter aspect indicated that these molecules could play important roles during development. More than 30 BMPs have been identified to date. The study of their expression pattern as well as the analysis of spontaneously mutated or genetically depleted mice have demonstrated a much broader range of function. These activities are mainly localized at sites of epithelial-mesenchymal interactions, including but not restricted to the skeleton. This review presents our current knowledge about the functions of BMPs during skeleton development as well as in many other biologic processes.

Secreted molecules are key regulators of developmental and regenerative processes, as they allow cells to communicate with each other and acquire their fate. In the last two decades, members of one subgroup of the transforming growth factor-β (TGF-β) superfamily of growth factors, termed generically as "bone morphogenetic protein" (BMP), have been recognized as critical in controlling multiple organogenetic processes. Comprising an ever-growing number of identified homologues, the BMPs represents almost one third of the TGF-β superfamily, with more than 30 members already described (Fig. 1). Individually, the members of this subfamily of secreted molecules are termed either BMPs, osteogenic proteins (OPs), cartilage-derived morphogenetic proteins (CDMPs), or growth and differentiation factors (GDFs). They have been classified into several subgroups according to their structural similarities (Fig. 1).

As extensive studies of their functions are being performed, it becomes increasingly evident that the name GDFs would be more accurate to describe this subfamily than the historical term of BMPs. This name was proposed by Urist et al in their pioneering work demonstrating that demineralized bone extracts could induce de novo bone formation when implanted in ectopic sites in rats [1–3]. This process, which recapitulates all of the events occurring during embryonic skeleton development [4–6], from the beginning has generated a lot of interest among bone biologists and developmental biologists. Indeed, in a sequence that is now very well known, the implant is first colonized by undifferentiated mesenchymal cells that differentiate into chondrocytes. Later on, vascular invasion occurs followed by osteoblast and osteoclast differentiation preceding de novo bone formation. These experiments performed in the 1960s were followed by the cloning of the first BMPs more than 20 years later, and the demonstration that these factors could reproduce the bone-forming activity of the bone extracts [7–12]. The molecular cloning of many BMP-encoding genes and their identification as TGF-β relatives enhanced the interest in these molecules and allowed expression and functional studies to be performed. Unexpectedly, given the nature of the assay that led to the identification of these proteins, it became rapidly evident that their pattern of expression, as well as their physiological functions, was not restricted to skeleton development when it affects skeleton development [13–16]. These functions include cell proliferation and differentiation; apoptosis; morphogenesis; patterning of various organs, including the skeleton; and organogenesis [17–19]. Moreover, recent experiments have shown that several BMP family members cannot induce de novo bone formation in the classic subcutaneous implantation assay [20], highlighting the functional heterogeneity of this subfamily. Taking this important fact in account, our review focuses not only on the few BMP family members whose ability to ectopically form bone in this assay has been demonstrated [10, 12, 21–25], but it also highlights what has been learned, using mostly findings of mouse and human genetics, about the nonskeletal function of particular BMPs in vivo.

Key words: transforming growth factor-β, organogenesis, bone formation, skeleton, gene mutation, development.

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STRUCTURE AND MECHANISM OF ACTION OF BMPs

Bone morphogenetic proteins are synthesized as large precursor proteins. Upon dimerization, they are proteo-

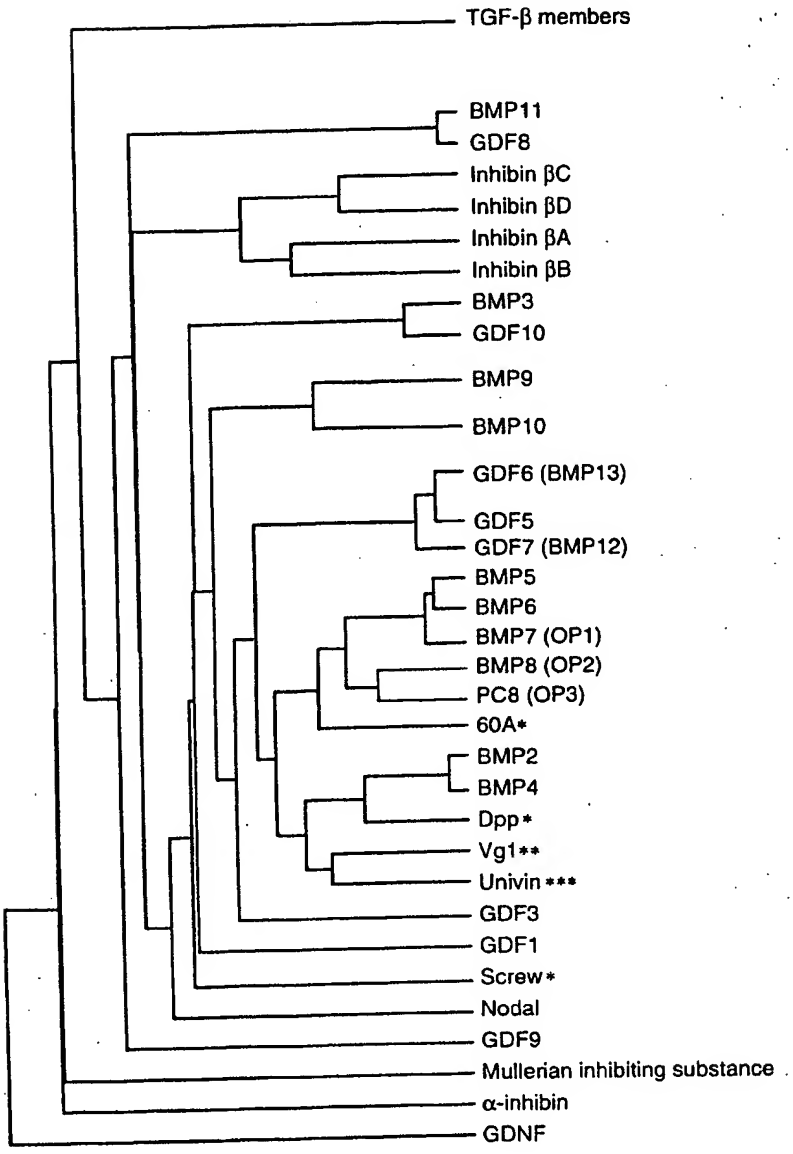

Fig. 1. Phylogenetic relationships between bone morphogenic protein (BMP) family members. Amino acid sequence identities were calculated based on the ligand-binding domain sequence, which is located downstream of the first conserved cysteine residue. The length of the horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences. No asterisk, human/mouse protein; **Drosophila* protein; ***Xenopus* protein; ***Sea urchin protein.

lytically cleaved at a consensus Arg-X-X-Arg site to yield carboxy-terminal mature dimers [7, 26, 27]. This cleavage is thought to occur before secretion and subtilisin-like convertases (SCPs) have been implicated in this process [28, 29]. It has been recently shown that the downstream sequence adjacent to the cleavage site determines the cleavage efficiency, while the N-terminal region controls the stability of the processed mature protein [29]. Analysis of the crystal structure of two TGF-β family members, TGF-β2 and BMP7, has revealed that the core of the monomer is a cystine knot involving six cysteine residues that are invariably spaced in the C-terminal region of all family members [30–32]. In general, BMPs have one more conserved cysteine that is involved in intermolecular disulfide bonding, but some members of the family, such as Gdf3 and Gdf9, lack this cysteine, suggesting that covalent bonding is not required for dimerization [33].

Once secreted, the BMPs fulfill their signaling function by binding to an heterodimeric complex of two transmembrane receptors, termed type I and type II, with serine-threonine kinase activity [15, 26, 34]. The kinase activity of the type II receptors is constitutive, while ligand binding is required for type I receptor kinase activation. Optimal ligand binding is achieved when both

type I and type II receptors are present, although BMPs can bind to each of them weakly and subsequently recruit the second subunit. Upon ligand binding, the type II receptor transphosphorylates the type I receptor at the juxtaposition region activating the type I kinase [35, 36]. The latter then phosphorylates members of the Smad family of transcription factors that are subsequently translocated to the nucleus, where they activate the expression of target genes in concert with other transcription factors or coactivators [37–39]. It is not yet clear whether Smads are able to recognize specific binding sites and can bind alone to DNA.

PATTERN OF EXPRESSION OF BMPs

If the expression pattern of a gene or a family of genes is a reflection of its potential function, one would have predicted, given their restricted but spectacular function in vitro, that the genes encoding the various BMPs would be expressed preferentially if not exclusively in mesenchymal condensations prefiguring the future skeleton, in developing bones, and in differentiated chondrocytes and/or osteoblasts. As it turns out, this is not the case for any of the BMPs for which the pattern of expression has been described. The most extensive studies are summarized later in this article and illustrate the point that developing skeleton is neither the only nor the major site of expression of the BMPs.

Bmp2 expression can be detected as early as 8.5 days postcoitum (dpc) in mesodermal cells of the amnion and chorion cells of the visceral endoderm, the allantois, and the lateral plate mesoderm underlying the head fold [40–42]. From 8.5 to 9.5, *Bmp2* is also expressed in the dorsal surface ectoderm underlying the neural tube [43]. Around 9.5 and 10.5 dpc, its expression can be detected in the outer myocardial layer of the heart, in the apical ectodermal ridge, and in the zone of polarizing activity of the developing limb [41–44]. Starting at 12.5 dpc, *Bmp2* expression is also observed in the mesenchymal condensation that will give rise to the ribs and vertebrae, in tooth buds, in the developing eye, and in whisker follicles [39, 43, 44]. Later during development, *Bmp2* transcript can be observed in hypertrophic chondrocytes of long bones and in forming digits [45].

Bmp4, which encodes a close relative to Bmp2, starts to be expressed as early as 6.5 dpc, around the time of gastrulation [46–48]. At 7.5 dpc, *Bmp4* becomes widely expressed in the embryo, marking the allantois, amnion, and the posterior part of the primitive streak [42, 46]. At that stage, this expression is restricted to ectodermal tissues in the chicken embryo [42]. Between 8.5 and 9.5 dpc, a strong *Bmp4* expression can be observed in the neural tube area, principally localized in the posterior mesoderm and prospective diencephalon, but also present in the dorsal surface ectoderm, and the presumptive neural crest cells [43]. As development proceeds, *Bmp4* transcripts become detectable in the mesoderm around the developing gut, in the myocardium, the branchial arches, the developing eye, the otic vesicles, the neuroepithelium that is destined to associate with Rathke's pouch, and the dorsomedial telencephalon region that will give rise to the choroid plexus [43, 46, 48–50]. As it is the case for *Bmp2*, *Bmp4* transcripts can be found in both the epithelium and mesenchyme tissues of the developing limb bud (mesoderm and apical ectodermal ridge) and tooth bud, suggesting that it also plays a role in the epithelial–mesenchymal interactions that characterize skeleton and tooth development, as well as many other organogenesis processes [46, 48].

Bmp5 is expressed in the mesenchymal cells of the developing lung starting at 10.5 dpc. Thereafter, it is expressed in the ureter and connective tissue-producing cells underlying the bladder in the presumptive gut, in the ventricular chamber of the heart, the meninges, and transiently in the telencephalon [43, 50, 51]. During skeletal development, *Bmp5* expression marks some of the mesenchymal condensations such as the genital tubercle, the sternum, the thyroid cartilage, the cartilage rings of the trachea, and the cells forming part of the vertebrae [45, 52, 53].

Bmp6 has also a broad pattern of expression. At 8.5 dpc, it is expressed in the branchial pouch and in the endodermal component of the visceral yolk sac [50, 54]. By 9.5 dpc, *Bmp6* transcripts can be detected in the roofplate of the neural tube [50]. In the developing heart, *Bmp6* expression is restricted to the epithelium of the branchial pouch [40, 46]. Moreover, *Bmp6* is expressed in the developing kidney, where it marks the stromal cells surrounding the developing and mature tubercles, and in the developing skin [39, 46]. In the developing skeleton, *Bmp6* is expressed preferentially in hypertrophic chondrocytes [45, 55].

Bmp7 is another member of the family whose broad expression has been extensively studied. It starts to be expressed during early gastrulation, localizing in the ectoderm of the periphery of the embryo [40, 42]. At 8.5 and 9.5 dpc, *Bmp7* is strongly expressed in the surface ectoderm and the notochord, in the neuroepithelium extending toward the prospective forebrain, and in the developing gut [40, 43]. *Bmp7* is also expressed in the atrial and ventricular chambers. This heart expression will continue throughout development [43]. During eye development, *Bmp7* expression is detected in the surface ectoderm, the lens placode, and the optic vesicle [40, 43, 56, 57]. In the developing skeleton, *Bmp7* is expressed in the developing limb, in the mesenchymal cells localized between the developing digits, and in the chondrogenic zones [43, 50, 56, 57]. *Bmp7* is also expressed in many cells of the developing kidney, where it plays a critical role (discussed later in this article). At 9.5 dpc, *Bmp7*

transcripts are detected in the Wolffian ducts, which give rise to the ureteric buds. At 12.5 dpc, *Bmp7* is expressed predominantly in the ureteric bud epithelium, but a weaker expression is also detected in the surrounding condensed mesenchymal cells. Later on, its transcripts are present in the same structures, but also in the pretubular aggregates and the podocytes of the glomeruli. No expression was detected in the comma- and S-shaped bodies [56, 57].

Other BMPs have not been shown to be expressed in developing skeletal structures, but seem to be specifically expressed in particular tissues. For instance, Gdf9 is expressed in the ovaries, testis, and hypothalamus [58–60], whereas Gdf8 expression is restricted to skeletal muscles [61], and BMP10 expression appears to be restricted to the heart [62]. Finally, several other BMPs are expressed more specifically in the developing nervous system, such as Gdf1, Gdf7, and Gdf11 [63–65].

From this long enumeration, a few common themes emerge. The expression of the BMPs is often widespread and dynamic as development proceeds. It is frequently localized at areas of epithelial–mesenchymal interactions. It is found in many more tissues and organs than the future skeleton, and sometime it interests the developing skeleton only marginally. Thus, from these few examples, one could predict that the various BMPs could have a broad range of physiologic functions during embryonic development and beyond development.

SKELETAL ABNORMALITIES IN BMP-DEFICIENT ANIMALS

Through identification of the mutated genes in classic mouse mutants or through conventional gene-targeting approaches, many BMP-encoding genes have been inactivated. The chronology of publication of the molecular elucidation of these various mutations led initially to the belief that BMPs were acting mostly on skeletal development. However, a careful review of all of the mutants available nowadays clearly indicates that this is an exception rather than a rule. We first review skeletal abnormalities in mice carrying a mutation in BMP-encoding genes and subsequently review the nonskeleton phenotypes.

Recessive short ear mouse

The recessive short ear (se/se) mouse is a classic skeleton-patterning mutant that has been very well studied over the last 60 years [66–71]. The short ear mouse has many anatomical abnormalities: reduced size of the ear, reduction in body size, reduction in the number of ribs, misshapen or absence of the xiphoid appendix as well as hydronephrosis, medial misplacement of the left gonad, giant cell granulomas of the liver, and lung abnormalities. Green, in a series of landmark studies, hypothesized that it was possible to relate the variety of the skeletal phenotypes observed in the short ear mice to a single function of the short ear gene during chondrocyte differentiation [68, 70, 71]. This hypothesis was supported by the finding, among others, that the abnormal shape or absence of the xiphoid appendix was due to a delay in the formation of the mesenchymal condensation prefiguring it. The original hypothesis proved to be extremely accurate, as it stated that the short ear gene causes "a slight reduction in the rate of formation of cartilage" but not an arrest of differentiation, as chondrocytes and osteoblasts are present, even in affected skeletal structures, in the short ear mice. This hypothesis could extend to the nonskeletal phenotypes only if one assumes that the "short ear gene" acts in multiple organs where it is expressed.

In a landmark study, 50 years after the hypothesis was proposed that the short ear gene was controlling cartilage formation, Kingsley et al demonstrated that the gene mutated or deleted in the short ear mice was the *Bmp5* gene [72]. These authors went on to show that *Bmp5* is expressed in the mesenchymal condensations outlining the future skeleton elements that are affected as well as in the periosteum [52]. Subsequently they showed that *Bmp5* is also expressed in lung, liver, bladder, and intestines, all organs that are affected in the short ear mice [52, 71]. A recent histomorphometric analysis revealed that the absence of BMP5 led to a minimal increase in growth plate height and growth rate, but no change in the number of proliferative and hypertrophic chondrocytes [73].

Brachypodism

Brachypodism (bp) is another classic mouse mutation that appears spontaneously on an outbred mouse strain in 1952 [74]. Affected animals are characterized by a reduction in length of several long bones and the replacement of two bones in most digits by a single skeleton element [74, 75]. The product of the *bp* gene was thought for a long time to act cell nonautonomously and to control the formation of mesenchymal condensations [76, 77]. A similar approach was taken by the same group that uncovered the involvement of *Bmp5* in the short ear mutation to demonstrate that the brachyphodism phenotype was due to frameshift mutations causing early termination in the *Gdf5* gene [78]. Gdf5 is a member of the BMP subfamily that does not have bone-forming activity but induces the formation of tendon and ligament structures in the classic subcutaneous implantation assay used to identify the BMPs [20]. Consistent with this pharmacological ability, *Gdf5* is expressed during joint formation in vivo [53, 79]. However, a mutation in the human *GDF5* gene called *CDMP1* has been implicated in two recessive chondrodysplasia: the Hunter Thompson chondrodysplasia and the chondrodysplasia Grebe type [80–82]. Mutations in the *CDMP1* gene also

cause autosomal dominant brachydactyly type C [83]. In in vitro studies, it was clear that overexpression of GDF5 increased the size of the mesenchymal condensations without a major effect on chondrocyte proliferation [76, 77, 84]. This example illustrates the need to perform in vivo experiments to understand the physiological function of a particular molecule accurately.

Another remarkable fact is that both *Bmp5* and *Gdf5* mutations in the same gene cause recessive phenotypes in mouse and dominant ones in humans. This seems to be a recurrent theme in the BMP field. Indeed, mice heterozygous for a deletion of noggin, an inhibitor of BMP action, have no overt phenotype whereas humans heterozygous for a similar mutation develop a multiple synostosis phenotype [85, 86].

Mutations in *Bmp5* and *Gdf5* were the first mutations to be identified in BMP-encoding genes, and they seemed to indicate that the biologic function of all BMP proteins could take place primarily if not only in the developing skeleton. As reported later in this article, subsequent mutagenesis experiments in other genes showed that it was not to be the case. In fact, the mutations published in BMP-encoding genes other than *Bmp5* or *Gdf5* have at best mild skeletal abnormalities. Homozygous *Bmp2* or *Bmp4* deletions in mice are embryonic lethal before the onset of skeletogenesis [41, 48]. Twelve percent of heterozygote *Bmp4*-deficient mice have a preaxial polydactyly [86]. Homozygous *Bmp6*-deficient mice have no skeleton-patterning defect and a mild delay of sternum ossification that could be traced to the formation of the mesenchymal condensations [45]. Homozygous *Bmp7*-deficient mice have patterning abnormalities of the ribs and a preaxial polydactyly in the hind limbs [56, 57]. As it is the case for the short ear mice, histologic examination failed to detect any other abnormalities in chondrocyte and osteoblast differentiation in the *Bmp7*-deficient mice [57].

The generation of mice harboring mutations in two different BMP-encoding genes adds little to this picture. *Bmp5/6* double mutant mice display only a slight exacerbation of the sternal defect present in single mutants [45]. Mice carrying null mutations in both *Gdf5* and *Bmp5* have skeleton-patterning defects that are not seen in any of the single mutants, but these abnormalities are extremely localized and do not disturb the differentiation of osteoblasts or chondrocytes [87]. Likewise, mice carrying heterozygous mutations in both *Bmp7* and *Bmp4* have a higher frequency of rib cage and digit abnormalities than single heterozygotes, suggesting that BMP7 and BMP4 may act jointly in the formation of the affected mesenchymal condensations [88]. Finally, *Bmp5/7* double mutant mice die early during embryogenesis, before the beginning of skeletogenesis [51].

The mild and/or extremely localized skeletal defects observed in all these mutant mice contrast strongly with the severity of the phenotype observed in other organs (discussed later in this article). It indicates that the physiologic functions of these proteins are different from their pharmacological ability in two critical ways. Physiologically, they control mesenchymal condensation but not cell differentiation, at least in the skeleton, and their actions in soft tissues appear to be more important than their actions in the skeleton.

EXTRASKELETAL ABNORMALITIES IN BMP-DEFICIENT ANIMALS

Consistent with their wide pattern of expression, mutations in BMP-encoding genes can affect many organogenetic processes. Some of these mutations cause early embryonic lethality, precluding for now the analysis of the role of these proteins during skeleton development. We review briefly some of these phenotypes, with a particular emphasis on BMP7, a gene product required for glomeruli formation [56, 57]. Again, the purpose of this enumeration is to highlight the difference between the wealth of their biologic function and the remarkable pharmacology of this group of molecules.

Inactivating mutation in some *Bmps* can result in early embryonic lethality or in perinatal lethality, caused by profound extraskeletal defects. For instance, *Bmp2*-deficient mice died between 7.0 and 10.5 dpc, the mutant phenotype being evident at 7.75 dpc [41]. The mutant embryo retained an open preamniotic canal and/or exhibited abnormal heart development. The maintenance of the preamniotic canal in an open state caused malformation of the amnion and the chorion. Heart development occurred abnormally in the exocoelonic cavity. Other abnormalities include delay in allantois development, open neural tubes, and overall slower growth of these embryos.

Inactivating mutations in *Bmp4* also causes embryonic lethality, this time between 6.5 and 9.5 dpc with a variable phenotype [48]. Most of the mutant embryos show virtually no mesodermal differentiation. Some embryos develop beyond 6.5 dpc and present disorganized posterior structures, a reduction in extraembryonic mesoderm, and are overall developmentally retarded. More recently, it was shown that they contain no primordial germ and that lens induction does not occur in these embryos [89, 90]. *Bmp4* heterozygote-deficient mice present with a variable penetrance craniofacial malformation, microphthalmia, and preaxial polydactyly, indicating that *Bmp4* gene dosage is essential for normal organogenesis. The extent and the severity of the abnormalities recorded in the *Bmp4* homozygous or heterozygous mutant embryos mice emphasize the pleiotropic function of the BMPs in vivo.

Another BMP-encoding gene in which the deletion causes a perinatal lethality in mice is *Bmp7* [56, 57].

In the absence of *Bmp7*, there is a failure of kidney morphogenesis, the deficient mice presenting small dysgenic kidneys that have less than 3 glomeruli per histologic section compared with approximately 100 in normal mice. This defect was tracked down to a defect in epithelial–mesenchymal interaction occurring between 12.0 and 12.5 dpc and leading to cell death of the metanephric mesenchymal cells. A series of molecular analyses suggest that Bmp7 is one of the earliest glomeruli inducers. Bmp7 deficiency also causes, with incomplete penetrance, an absence of lens induction and eye formation. This latter phenotype is reminiscent to what is observed in *Bmp4*-deficient embryos. This is important as it suggests that, at least during lens formation, redundancy between BMPs may not prevent abnormalities to occur.

As Green pointed out, *Bmp5*-deficient mice (se/se) have also many nonskeletal phenotypes, although none of them are life threatening [71]. Those can include medial displacement of the left gonad, ventralization of the right renal artery, hydronephrosis, giant cell granuloma on the ventral surface of the liver, and cysts of the lung. These defects are all in agreement with the pattern of expression of *Bmp5* [52]. The molecular basis of the hydronephrosis of the se/se mice has not yet been elucidated.

Finally, the increasing number of BMP-encoding genes in which inactivation in mice did not give any visible skeletal abnormalities while other organs or functions where profoundly affected cannot be ignored. For instance, deficiency in GDF7, a close relative of GDF5 [78], blocks the generation of a particular class of neurons [64]. Considering the ligament/tendon-forming activity of GDF7 in the subcutaneous implantation assay [20], such a role was unexpected. This result, along with the expression of *Bmp2*, *Bmp4*, *Bmp7*, and *Gdf1* and *Gdf11* in the developing brain [43, 50, 63, 65], indicates that BMPs may play an important, yet poorly understood role in the morphogenesis of central nervous system. Inactivation of *Gdf8*, also called myostatin, induced in mice or spontaneous in bovine, results in muscular hypertrophy, implicating BMPs in the control of skeletal muscle growth [61, 91]. Germ cell proliferation and maturation are other functions that BMPs seem to play a critical role. *Bmp8a* and *Bmp8b* are tightly linked on mouse chromosome 4 and have a similar pattern of expression. Inactivation of *Bmp8a* leads to germ cell degeneration in nearly 50% of the adult males [92]. Likewise *Bmp8b*-deficient mice have as a sole reported phenotype, a male germ-cell deficiency and sterility [93]. These two studies identified these two BMPs as critical proteins in germ-cell development, maintenance of spermatogenesis, and fertility in male mice. BMPs are also involved in maintenance of the female reproductive system. Female mice null for *Gdf9*, an oocyte-specific gene, exhibit primary infertility caused by failed ovarian follicular development [59, 60, 94].

In summary, genetic and molecular studies have helped define the paramount importance of the BMPs in multiple physiologic processes. In fact, it is now clear that most of the BMPs studied so far have, in vivo, profound and specific effects on organogenesis processes outside the skeleton. Because some of these effects lead to embryonic lethality, it is possible that additional roles may have been underestimated. The possibility now exists to perform cell-specific and time-specific experiments on genes at will. Most likely, when they are performed these studies will determine the extent of the physiologic role of the BMPs during development.

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REFERENCES

1. Urist MR: Bone: Formation by autoinduction. *Science* 150:893–899, 1965
2. Urist MR, Iwata H, Ceccotti PL, Dorfman RL, Boyd SD, McDowell RM, Chien C: Bone morphogenesis in implants of insoluble bone gelatin. *Proc Natl Acad Sci USA* 70:3511–3515, 1973
3. Urist MR, Mikulski A, Lietze A: Solubilized and insolubilized bone morphogenetic protein. *Proc Natl Acad Sci USA* 76:1828–1832, 1979
4. Reddi AH: Regulation of cartilage and bone differentiation by bone morphogenetic proteins. *Curr Opin Cell Biol* 4:850–855, 1992
5. Horton WA: Morphology of connective tissue: Cartilage, in *Connective Tissue and Its Heritable Disorders*, New York, Wiley-Liss, Inc., 1992, pp 73–84
6. Reddi AH: Bone and cartilage differentiation. *Curr Opin Genet Dev* 4:737–744, 1994
7. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA: Novel regulators of bone formation: Molecular clones and activities. *Science* 242:1528–1534, 1988
8. Sampath TK, Muthukumaran N, Reddi AH: Isolation of osteogenin, an extracellular matrix-associated bone-inductive protein, by heparin affinity chromatography. *Proc Natl Acad Sci USA* 84:7109–7113, 1987
9. Wang EA, Rosen V, Cordes P, Hewick RM, Kriz MJ, Luxenberg DP, Sibley BS, Wozney JM: Purification and characterization of other distinct bone-inducing factors. *Proc Natl Acad Sci USA* 85:9484–9488, 1988
10. Luxenberg DP, McQuaid D, Moustatsos IK, Nove J, Wozney JM: Recombinant human bone morphogenetic protein induces bone formation. *Proc Natl Acad Sci USA* 87:2220–2224, 1990
11. Wang EA, Wozney JM: Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. *Proc Natl Acad Sci USA* 87:9843–9847, 1990
12. Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RH: Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. *J Biol Chem* 267:20352–20362, 1992
13. Wozney JM: The bone morphogenetic protein family and osteogenesis. *Mol Reprod Dev* 32:160–167, 1992
14. Harland RM: The transforming growth factor β family and induction of the vertebrate mesoderm: Bone morphogenetic proteins are ventral inducers. *Proc Natl Acad Sci USA* 91:10243–10246, 1994
15. Hogan BLM: Bone morphogenetic proteins: Multifunctional regulators of vertebrate development. *Genes Dev* 10:1580–1594, 1996
16. Reddi AH: Bone morphogenetic proteins: An unconventional

approach to isolation of first mammalian morphogens. *Cytokine Growth Factor Rev* 8:11–20, 1997
17. Graff JM: Embryonic patterning: To BMP or not to BMP, that is the question. *Cell* 89:171–174, 1997
18. Ebendal T, Bengtsson H, Soderstrom S: Bone morphogenetic proteins and their receptors: Potential functions in the brain. *J Neurosci Res* 51:139–146, 1998
19. Wozney JM: The bone morphogenetic protein family: Multifunctional cellular regulators in the embryo and adult. *Eur J Oral Sci* 106:160–166, 1998
20. Wolfman NM, Hattesley G, Cox K, Celeste AJ, Nelson R, Yamaji N, Dube JL, Diblasio-Smith E, Nove J, Song JJ, Wozney JM, Rosen V: Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family. *J Clin Invest* 100:321–330, 1997
21. Hammonds RG Jr, Schwall R, Dudley A, Berkemeier L, Lai C, Lee J, Cunningham N, Reddi AH, Wood WI, Mason AJ: Bone-inducing activity of mature BMP-2b produced from a hybrid BMP-2a/2b precursor. *Mol Endocrinol* 5:149–155, 1991
22. Cox K, Holtrop M, D'Alessandro JS, Wang EA, Wozney JM, Rosen V: Histological and ultrastructural comparison of the in vivo activities of rhBMP-2 and rhBMP-5. *J Bone Miner Res* 6:S155, 1991
23. D'Alessandro JS, Cox KA, Israel DI: Purification, characterization and activities of recombinant bone morphogenetic protein 5. *J Bone Miner Res* 6:S153, 1991
24. Gitelman SE, Kobrin MS, Ye J-Q, Lopez AR, Lee A, Derynck R: Recombinant Bgr-1 BMP-6-expressing tumors induce fibrosis and endochondral bone formation in vivo. *J Cell Biol* 126:1595–1609, 1994
25. Rutherford RB, Sampath TK, Rueger DC, Taylor TD: Use of bovine osteogenic protein to promote rapid osseointegration of endosseous dental implants. *Int J Oral Maxillofac Implants* 7:297–301, 1992
26. Kingsley DM: The TGF-β superfamily: New members, new receptors, and genetic tests of function in different organisms. *Genes Dev* 8:133–146, 1994
27. Israel DI, Nove J, Kerns KM, Moutsatsos IK, Kaufman RJ: Expression and characterization of bone morphogenetic protein-2 in Chinese hamster ovary cells. *Growth Factors* 7:139–150, 1992
28. Cui Y, Jean F, Thomas G, Christian JL: BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development. *EMBO J* 17:4735–4743, 1998
29. Constam DB, Robertson EJ: Regulation of bone morphogenetic protein activity by pro domains and proprotein convertases. *J Cell Biol* 144:139–149, 1999
30. Schlunegger MP, Grutter MG: An unusual feature revealed by the crystal structure at 2.2: A resolution of human transforming growth factor-beta 2. *Nature* 358:430–434, 1992
31. Daopin S, Piez KA, Ogawa Y, Davies DR: Crystal structure of transforming growth factor-beta 2: An unusual fold for the superfamily. *Science* 257:369–373, 1992
32. Griffith DL, Keck PC, Sampath TK, Rueger DC, Carlson WD: Three-dimensional structure of recombinant human osteogenic protein 1: Structural paradigm for the transforming growth factor beta superfamily. *Proc Natl Acad Sci USA* 93:878–883, 1996
33. McPherron AC, Lee SJ: GDF-3 and GDF-9: Two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. *J Biol Chem* 268:3444–3449, 1993
34. Massague J, Weis-Garcia F: Serine/threonine kinase receptors: Mediators of transforming growth factor beta family signals. *Cancer Surv* 27:41–64, 1996
35. Liu F, Ventura F, Doody J, Massague J: Human type II receptor for bone morphogenic proteins (BMPs): Extension of the two-kinase receptor model to the BMPs. *Mol Cell Biol* 15:3479–3486, 1995
36. Ruberte E, Marty T, Nellen D, Affolter M, Basler K: An absolute requirement for both the type II and type I receptors, punt and thick veins, for dpp signaling in vivo. *Cell* 80:889–897, 1995
37. Heldin CH, Miyazono K, ten Duke P: TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 390:465–471, 1997
38. Massague J: TGF-beta signal transduction. *Annu Rev Biochem* 67:753–791, 1998
39. Lyons KM, Pelton RW, Hogan BL: Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-beta-like genes coordinately regulate aspects of embryonic development. *Genes Dev* 3:1657–1668, 1989
40. Lyons KM, Hogan BL, Robertson EJ: Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. *Mech Dev* 50:71–83, 1995
41. Zhang H, Bradley A: Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development* 122:2977–2986, 1996
42. Schultheiss TM, Burch JBE, Lassar AB: A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev* 11:451–462, 1997
43. Dudley AT, Robertson EJ: Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. *Dev Dyn* 208:349–362, 1997
44. Lyons KM, Pelton RW, Hogan BL: Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). *Development* 109:833–844, 1990
45. Solloway MJ, Dudley AT, Bikoff EK, Lyons KM, Hogan BL, Robertson EJ: Mice lacking Bmp6 function. *Dev Genet* 22:321–339, 1998
46. Jones CM, Lyons KM, Hogan BL: Involvement of bone morphogenetic protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. *Development* 111:531–542, 1991
47. Johansson BM, Wiles MV: Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and haematopoietic development. *Mol Cell Biol* 15:141–151, 1995
48. Winnier G, Blessing M, Labosky PA, Hogan BLM: Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev* 9:2105–2116, 1995
49. Oh SH, Johnson R, Wu DK: Differential expression of bone morphogenetic proteins in the developing vestibular and auditory sensory organs. *J Neurosci* 16:6463–6475, 1996
50. Furuta Y, Piston DW, Hogan BL: Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* 124:2203–2212, 1997
51. Solloway MJ, Robertson EJ: Early embryonic lethality in Bmp5;Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. *Development* 126:1753–1768, 1999
52. King JA, Marker PC, Seung KJ, Kingsley DM: BMP5 and the molecular, skeletal, and soft-tissue alterations in *short ear* mice. *Dev Biol* 166:112–122, 1994
53. Storm EE, Kingsley DM: GDF5 coordinates bone and joint formation during digit development. *Dev Biol* 209:11–27, 1999
54. Farrington SM, Belaoussoff M, Baron MH: Winged-helix, Hedgehog and BMP genes are differentially expressed in distinct cell layers of the murine yolk sac. *Mech Dev* 62:197–211, 1997
55. Iwasaki M, Le AX, Helms JA: Expression of *Indian hedgehog, bone morphogenetic protein 6* and *gli* during skeletal morphogenesis. *Mech Dev* 69:197–202, 1997
56. Dudley A, Lyons K, Robertson EJ: A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev* 9:2795–2807, 1995
57. Luo G, Hofmann C, Bronckers ALJ, Sohocki M, Bradley A, Karsenty G: BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev* 9:2808–2820, 1995
58. McGrath SA, Esquela AF, Lee SJ: Oocyte-specific expression of growth/differentiation factor-9. *Mol Endocrinol* 9:131–136, 1995
59. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM: Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* 383:531–535, 1996
60. Fitzpatrick SL, Sindoni DM, Shughrue PJ, Lane MV, Merchenthaler IJ, Frail DE: Expression of growth differentiation factor-9 messenger ribonucleic acid in ovarian and nonovarian rodent and human tissues. *Endocrinology* 139:2571–2578, 1998
61. McPherron AC, Lawler AM, Lee SJ: Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 387:83–90, 1997

62. Neuhaus H, Rosen V, Thies RS: Heart specific expression of mouse BMP-10 a novel member of the TGF-beta superfamily. *Mech Dev* 80:181–184, 1999
63. Lee SJ: Expression of growth/differentiation factor 1 in the nervous system: Conservation of a bicistronic structure. *Proc Natl Acad Sci USA* 88:4250–4254, 1991
64. Lee KJ, Mendelsohn M, Jessell TM: Neuronal patterning by BMPs: A requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev* 12:3394–3407, 1998
65. Nakashima M, Toyono T, Akamine A, Joyner A: Expression of growth/differentiation factor 11, a new member of the BMP/TGFβ superfamily during mouse embryogenesis. *Mech Dev* 80:185–189, 1999
66. Lynch CJ: Short ears, an autosomal mutation in the house mouse. *Am Nat* 55:421–426, 1921
67. Green EL, Green MC: The development of three manifestations of the short ear gene in the mouse. *J Morphol* 70:1–19, 1942
68. Green EL, Green MC: Effect of the short ear gene on number of ribs and presacral vertebrae in the house mouse. *Am Nat* 80:619–625, 1946
69. Green MC: Further morphological effects of the short ear gene in the house mouse. *J Morphol* 88:1–22, 1951
70. Green MC: Effects of the short ear gene in the mouse on cartilage formation in healing bone fractures. *J Exp Zool* 137:75–88, 1958
71. Green MC: Mechanism of the pleiotropic effects of the short ear mutant gene in the mouse. *J Exp Zool* 167:129–150, 1968
72. Kingsley DM, Bland AE, Grubber JM, Marker PC, Russell LB, Copeland NG, Jenkins NA: The mouse short ear skeletal morphogenesis locus is associated with defects in a bone morphogenetic member of the TGF beta superfamily. *Cell* 71:399–410, 1992
73. Mikic B, Van der Meulen MC, Kingsley DM, Carter DR: Mechanical and geometric changes in the growing femora of BMP-5 deficient mice. *Bone* 18:601–617, 1996
74. Landauer W: Brachypodism, a recessive mutation of house mice. *J Hered* 43:293–298, 1952
75. Gruneberg H, Lee AJ: The anatomy and development of brachypodism in the mouse. *J Embryol Exp Morphol* 30:119–141, 1973
76. Malinina NA, Kindiakov BN, Koniukhov BV: Mutant gene expression in mouse aggregation chimeras. 3. Barachypodism-H gene. *Ontogenes* 15:514–521, 1984
77. Owens EM, Solursh M: Cell-cell interaction by mouse limb cells during in vitro chondrogenesis: Analysis of the brachypod mutation. *Dev Biol* 91:376–388, 1982
78. Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, Lee S-J: Limb alterations in *brachypodism* mice due to mutations in a new member of the TGFβ-superfamily. *Nature* 368:639–643, 1994
79. Merino R, Macias D, Ganan Y, Economides AN, Wang X, Wu Q, Stahl N, Sampath KT, Varona P, Hurle JM: Expression and function of Gdf-5 during digit skeletogenesis in the embryonic chick leg bud. *Dev Biol* 206:33–45, 1999
80. Langer LO Jr, Cervenka J, Camargo M: A severe autosomal recessive acromesomelic dysplasia, the Hunter-Thompson type, and comparison with the Grebe type. *Hum Genet* 81:323–328, 1989
81. Thomas JT, Lin K, Nandedkar M, Camargo M, Cervenka J, Luyten FP: A human chondrodysplasia due to a mutation in a TGF-beta superfamily member. *Nat Genet* 12:315–317, 1996
82. Thomas JT, Kilpatrick MW, Lin K, Erlacher L, Lembessis P, Costa T, Tsipouras P, Luyten FP: Disruption of human limb morphogenesis by a dominant negative mutation in DCMP1. *Nat Genet* 17:58–64, 1997
83. Polinkovsky A, Robin NH, Thomas JT, Irons M, Lynn A, Goodman FR, Reardon W, Kant SG, Brunner HG, van der Burgt I, Chitayat D, McGaughran J, Donnai D, Luyten FP, Warman ML: Mutations in CDMP1 cause autosomal dominant brachydactyly type C. *Nat Genet* 17:18–19, 1997
84. Francis-West PH, Abdelfattah A, Chen P, Allen C, Parish J, Ladher R, Allen S, MacPherson S, Luyten FP, Archer CW: Mechanisms of GDF-5 action during skeletal development. *Development* 126:1305–1315, 1999
85. Brunet LJ, McMahon JA, McMahon AP, Harland RM: Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* 280:1455–1457, 1998
86. Gong Y, Krakow D, Marcelino J, Wilkin D, Chitayat D, Babul-Hirji R, Hudgins L, Cremers CW, Cremers FP, Brunner HG, Reinker K, Rimoin DL, Cohn DH, Goodman FR, Reardon W, Patton M, Francomano CA, Warman ML: Heterozygous mutations in the gene encoding noggin affect human joint morphogenesis. *Nat Genet* 21:302–304, 1999
87. Storm EE, Kingsley DM: Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. *Development* 122:3969–3979, 1996
88. Takenobu K, Boorla S, Frewndo J-L, Hogan BLM, Karsenty G: Skeletal abnormalities in doubly heterozygous *Bmp4* and *Bmp7* mice. *Dev Genet* 22:340–348, 1998
89. Furuta Y, Hogan BLM: BMP4 is essential for lens induction in the mouse embryo. *Genes Dev* 12:3764–3775, 1998
90. Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AN, Wright CV, Korving JP, Hogan L: Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev* 13:424–436, 1999
91. Kambadur R, Sharma M, Smith TP, Bass JJ: Mutations in myostatin (GDF8) in double-muscled Belgian blue and piedmontese cattle. *Genome Res* 7:910–916, 1997
92. Zhao GQ, Liaw L, Hogan BL: Bone morphogenetic protein 8A plays a role in the maintenance of spermatogenesis and the integrity of the epididymis. *Development* 125:1103–1112, 1998
93. Zhao GQ, Deng K, Labosky PA, Liaw L, Hogan BL: The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes Dev* 10:1657–1669, 1996
94. Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF: Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. *Dev Biol* 204:373–384, 1998

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Osteogenic Protein-2

A NEW MEMBER OF THE TRANSFORMING GROWTH FACTOR-β SUPERFAMILY EXPRESSED EARLY IN EMBRYOGENESIS*

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Osteogenic protein-2, OP-2, a new member of the transforming growth factor-β (TGF-β) superfamily, closely related to the osteogenic/bone morphogenetic proteins, was discovered in mouse embryo and human hippocampus cDNA libraries. The TGF-β domain of OP-2 shows 74% identity to OP-1, 75% to Vgr-1, and 76% to BMP-5, hence OP-2 may also have bone inductive activity. The genomic locus of OP-2 has seven exons, like OP-1, and spans more than 27 kilobases (kb). In the C-terminal TGF-β domain, OP-2 has a unique additional cysteine. Mouse embryos express relatively high levels of OP-2 mRNA at 8 days, two species of 3 and 5 kb. A careful study of mRNA expression of the osteogenic proteins in specific organs revealed discrete mRNA species for BMP-3, BMP-4, BMP-5, and BMP-6/Vgr-1 in lung or liver of young and adult mice. OP-1 is expressed in kidney; however, OP-2 and BMP-2 mRNAs were not detected in any organs studied, suggesting an early developmental role.

The extended TGF-β[1] superfamily is widely represented in vertebrates and invertebrates. Examples among vertebrates are the Vg-1 gene product of *Xenopus laevis* (1), Müllerian inhibiting substance (MIS) (2), the inhibins and activins (3–5) and bone morphogenetic proteins (BMP-2, -3, -4, and -5) (6, 7), Vgr-1 (8), and OP-1 (9). More distantly related to these is GDF-1 (growth and differentiation factor-1) (10). Representing the invertebrates are the decapentaplegic protein (DPP) and 60A gene product of *Drosophila melanogaster* (11, 12). Recently, receptors for activin and TGF-β were cloned and identified as serine threonine kinases (13–15).

TGF-β-related proteins are secreted as a pro form, with the C-terminal domain yielding dimeric mature protein (16, 25). The C-terminal TGF-β domain is characterized by conserved residues including a pattern of 7 cysteines.

In their biological roles, these proteins act during embryogenesis and in adult animals. For example, the DPP gene product is a determinant of the dorsal-ventral pattern formation of the fly, and null mutations have been shown to be lethal (17). Inhibins and activins control secretion of follicle-stimulating hormone (3, 5). Moreover, activins A and B show mesoderm-inducing activity (18–20). BMP-4 also induced mesoderm in a similar assay (21). MIS causes regression of the Müllerian duct during male development (2, 22). Recombinant BMP-2 (23), BMP-4 (24), and OP-1 (25) induce new bone formation in a subcutaneous implant assay in rats (26). Recombinant OP-1 homodimers, produced in mammalian cell culture, have been effective in the repair of segmental bone defects in rabbit ulna (25, 27).

A major site of OP-1 expression was found to be the kidneys (28). Expression of a bone growth factor in the kidneys is consistent with their role in calcium regulation and bone homeostasis. The original source of osteogenic protein, the bone tissue, contains several TGF-β-related proteins as evidenced by protein sequence analysis of purified osteogenic activity (6, 29). The OP-1 gene and other BMPs were cloned using a consensus probe, based on partial protein sequence information and conserved elements of the TGF-β family (9). Screening of cDNA libraries with OP-1 probe led to discovery of OP-2, which is described here.

MATERIALS AND METHODS

Library Screening—All libraries were screened by an initial plating of 1×10^6 plaques (approximately 5×10^4 plaques/plate) and hybridizations were in 40% formamide, 5 × SSPE, 5 × Denhardt's solution, and 0.1% SDS at 37 °C. Nonspecific counts were removed in 0.1 × SSPE, 0.1% SDS by shaking at 50 °C. Murine OP-2 cDNA was found in a 17-day mouse embryo 5′-stretch cDNA library (λ gt10, ML1029a, Clontech, Palo Alto, CA) and also in a teratocarcinoma (PCC4) cDNA library (ZAPII, 936301, Stratagene, La Jolla, CA). The complete human OP-2-coding sequence was derived from a cDNA clone in a human hippocampus cDNA library (λ ZAPII, 936205, Stratagene; from brain of a normal 2-year-old girl) and a genomic clone (EMBL-3 library, HL1067J, Clontech). The latter library also yielded the other clones used to determine the human OP-2 genomic locus. The genomic structure of human OP-1 was determined using two different libraries (Clontech, HL1067J; Stratagene, 946203). For identification of the splice junctions of OP-1 the following subclones were prepared. Exon 1 of OP-1, with both splice junctions, was subcloned as a 1.7-kb *Pst*I fragment (pO142-12) and exon 2, with splice junctions, was cloned as a 0.6-kb *Hae*III fragment (pO247-5). Part of the third exon and upstream junction was cloned as a 1.7-kb *Pst*I fragment (pO243), whereas the downstream junction was cloned as a 0.3-kb *Sau*3AI fragment (pO248). Exon 4, with splice junctions, was subcloned as a 0.7-kb *Pst*I fragment (pO229). Exons 4, 5, 6, and 7 were all present in the original OP-1 genomic clone (λ 13) (9).

DNA Sequencing—All sequencing was done according to Sanger *et al.* (30) using exonuclease III-mediated unidirectional deletion (31),

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M97016 (human OP-2) and M97017 (murine OP-2).

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[1] The abbreviations used are: OP-1, osteogenic protein-1; OP-2, osteogenic protein-2; Vgr-1, Vg-1 related protein-1; BMP, bone morphogenetic protein; GDF-1, growth/differentiation factor-1; MIS, Müllerian-inhibiting substance; DPP, decapentaplegic protein; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s).

subcloning of restriction fragments, and synthetic primers. The coding regions of human and murine OP-2 DNA were sequenced on both strands. Compressions were resolved by performing reactions at 70 °C with Taq polymerase and using 7-deaza-GTP (U. S. Biochemical Corp.).

Preparation of RNA and Northern Blot Analysis—Mice, strain CD-1, and rats (Long-Evans) were from Charles River Laboratories, Wilmington, MA. Total RNA from mice was isolated by the acid guanidine thiocyanate-phenol-chloroform method (32). Poly(A)$^+$ RNA was analyzed on 1.2% agarose-formaldehyde gels and blotted onto Nytran membranes (Schleicher & Schuell) with 10 × SSC (32). Hybridization conditions with ^{32}P-labeled (33) probes were as described (28). Between hybridizations filters were deprobed in 1 mM Tris-HCl, 1 mM EDTA, 0.1% SDS, pH 7.5, at 90–95 °C and exposed to film to assure complete removal of probe.

Cloning of Vgr-1 and GDF-1 cDNA and BMP-5-specific cDNA Fragments—A partial Vgr-1 clone, lacking some 300 nucleotides at the 5′-coding region was isolated from a mouse brain cDNA library (Clontech, ML1036a) using an OP-1 cDNA probe. A murine GDF-1 cDNA spanning the complete coding region was isolated by PCR. Oligo(dT) was used to prime first-strand synthesis from 200 ng of mouse brain poly(A)$^+$ RNA. Reactions were in a 100-μl volume with 5 μl of 20 × PCR reaction buffer (1 M Tris-HCl, pH 9.0, 400 mM ammonium sulfate, 30 mM magnesium chloride), 2 μl of 10 mM dNTPs, 100 pmol of oligo(dT), 200 units of Moloney murine leukemia virus reverse transcriptase for 60 min at 37 °C. Reactions were incubated for an additional 30 min at 37 °C with 5 units of RNase H (GIBCO-BRL) followed by 5 min at 95 °C. For amplification, 5′-GDF (GCGCAAGCTTGGACACCTCCTGGGAGG) and 3′-GDF (GGAATTCCTCAACGGCAGCCACACTCATC) primers were added to 0.5 μM. The cDNAs with 2.5 units of Replinase (Du Pont-New England Nuclear) were subjected to 1 cycle of denaturation at 94 °C for 2 min, annealing at 45 °C for 2 min, polymerization at 72 °C for 20 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and polymerization at 72 °C for 2 min. For mouse BMP-5, a 287-bp fragment was isolated by PCR from mouse embryo mRNA under similar conditions as described for GDF-1. A 3′-BMP-5-specific PCR primer (CCATGTCAGCATCATTCAG) was used for first strand synthesis. A 5′-BMP-5-specific PCR primer (CCAGACCATTTTCACCTG) was added for PCR amplification. PCR fragments were gel purified and cloned into a Bluescript KS(−) vector.

RESULTS

Cloning of Murine and Human OP-2 cDNA—In an effort to isolate additional OP-1 related genes, we screened several mouse cDNA libraries, using an OP-1 probe derived from the TGF-β domain (0.32-kb *Stu*I-*Eco*RI fragment). A 17-day mouse embryo cDNA library yielded not only the murine homolog of the human OP-1 gene (28) but also the new gene, termed OP-2. Only one OP-2 clone was found compared to four of OP-1, indicating a low abundance at the 17-day stage. A murine OP-2 clone was also found in a teratocarcinoma (PCC4) cDNA library.

The human OP-2 gene was isolated from a hippocampus cDNA library, previously the source of human OP-1 cDNA, by screening with murine OP-2 probe specific for the pro-region (0.3-kb *Eco*RI-*Bam*HI fragment). A positive clone (λ O24 and subclone pO166) shared extensive sequence homology with murine OP-2. It contained 0.4 kb of 5′-noncoding sequences, the complete pro-region, and the first half of the TGF-β domain but lacked 0.14 kb from the C terminus. The last portion of the TGF-β domain was obtained from a human genomic library (EMBL-3, Clontech): λ O28 contained the last four exons of human OP-2 and provided the missing part of the TGF-β domain on a *Bam*HI-*Pst*I fragment of 0.8 kb (pO173-2). Analysis of exons 4, 5, and 6 from this genomic clone revealed nucleotide sequences identical to the respective regions in the human cDNA. Subsequently, the entire human genomic OP-2 locus was isolated.

Based on cDNA and genomic DNA sequence, the predicted size for human pre-pro OP-2 is 402 amino acids (Fig. 1). The putative secretion signal peptide contains 19 amino acids and

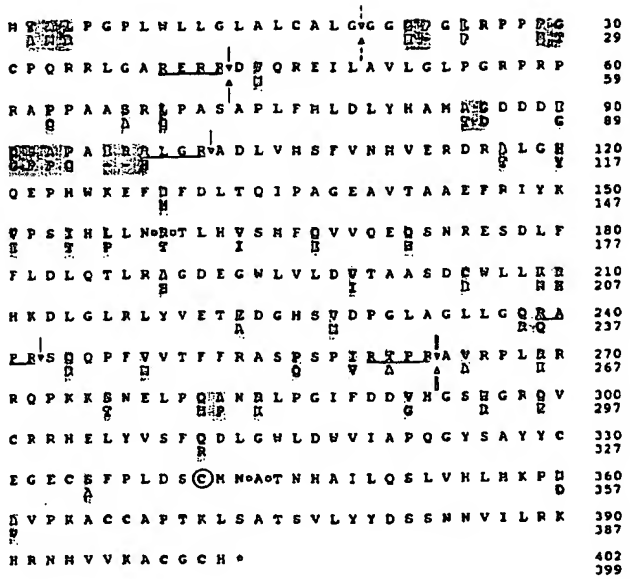

FIG. 1. **Deduced amino acid sequence of human and murine pre-pro OP-2.** The deduced amino acid sequence of human OP-2 is shown above variant amino acids of the murine OP-2. The *shaded boxes* emphasize these variant residues. Residue numbers of human and murine OP-2 are marked on the *right margin*. The proposed signal peptidase cleavage site and multiple RXXR maturation sites are shown by *vertical arrows*. Cysteine residues of mature OP-2 are shown in *bold letters*. The OP-2-specific cysteine residue is *circled*. Three *dashes* in the murine OP-2 sequence serve alignment. Potential *N*-glycosylation sites are marked by ✪.

the pro-protein contains 383 amino acids. The sequence RTPR↓A is proposed as the maturation cleavage site of OP-2, alanine being the first amino acid of the mature protein. Therefore, the N terminus of the mature protein, up to the first cysteine in the TGF-β domain, comprises 37 aa with mature OP-2 being 139 amino acids in length, like OP-1 and Vgr-1.

Alignments of the TGF-β Domains of OP-2 and Related Proteins—A distinctive feature of OP-2 is the presence of an additional cysteine in the TGF-β domain, which corresponds to a tyrosine residue in OP-1. OP-2 is the only TGF-β-related protein that breaks the pattern of 7 conserved cysteines in the TGF-β domain, as seen in the alignment with OP-1, BMP-5, Vgr-1, BMP-2, BMP-3, BMP-4, DPP, 60A, Vg-1, GDF-1, activins A and B, and TGF-β1 (Fig. 2). This alignment places OP-2 near OP-1, Vgr-1, BMP-5, and also 60A. Similarities, as percentage of identical amino acids, are compiled in Table I. OP-2 is equally related (in its TGF-β domain) to OP-1 (74%), Vgr-1 (BMP-6) (75%), and BMP-5 (76%) and also very close to the *Drosophila* 60A protein (65%). It is notably more distant from BMP-2 and BMP-4 (55%), which have arginine rather than histidine at the C terminus (Fig. 2). A glycosylation site in the center of the TGF-β domain is shared by OP-2, OP-1, BMP-5, BMP-6, 60A, DPP, BMP-2, and BMP-4, but absent in BMP-3, and more distantly related proteins.

Comparison of Human and Murine OP-2—Members of the TGF-β superfamily typically show extensive conservation across species. The alignment of human and mouse OP-2 in (Fig. 1) shows only 4 amino acid changes in the TGF-β domain while the mature proteins differ by 13 amino acids. In the pro-region, murine OP-2 is 3 amino acids shorter than human OP-2. Some diversion is found near residue 90 of OP-2, where OP-2 and OP-1 diverged the most (Fig. 3). Overall, a total of 59 aa changes between human and murine pre-pro OP-2

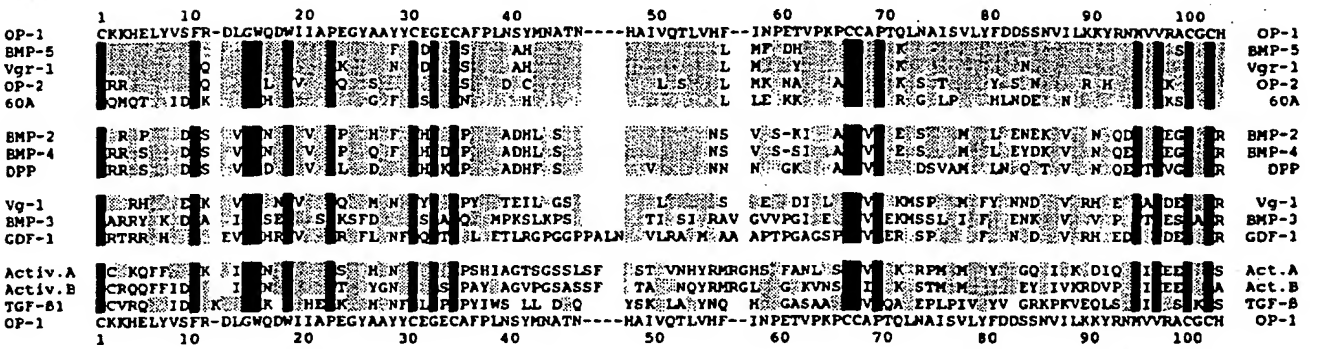

FIG. 2. **Sequence alignments of 7-cysteine domains.** The amino acid sequence of OP-1 is aligned with that of BMP-5, BMP-6/Vgr-1, OP-2, *Drosophila* 60A, BMP-2, BMP-4, DPP, Vg-1, BMP-3, GDF-1, activin A, activin B, TGF-β1. *Black bars* indicate complete conservation among all sequences and the shaded areas indicate partial conservation. Dashes (-) indicate gaps introduced for alignment.

TABLE I

Identities between the 7-cysteine-TGF-β domains

The percent identities in the 7-cysteine domains, which are aligned in Fig. 2, are arranged in this matrix as groups of similarity, separated by double lines.

	OP-2	OP-1	Vgr -1	BMP -5	60A	BMP -2	BMP -4	DPP	Vg-1	Act. A	BMP -3	Act. B	TGF β1
OP-2		75	75	76	65	55	55	53	55	39	40	36	38
OP-1	75		88	88	69	60	58	58	57	44	42	39	34
Vgr-1	75	88		92	71	62	59	58	59	45	44	39	35
BMP-5	76	88	92		72	59	57	52	55	44	42	39	33
60A	65	69	71	72		58	53	53	50	38	40	37	33
BMP-2	55	60	62	59	58		92	74	58	45	49	44	32
BMP-4	55	58	59	57	53	92		76	56	43	48	44	31
DPP	53	58	58	52	53	74	76		48	44	43	40	36
Vg-1	55	57	59	55	50	58	56	48		47	49	39	36
Activ.A	39	44	45	44	38	45	43	44	47		37	64	39
BMP-3	40	42	44	42	40	49	48	43	49	37		38	32
Activ.B	36	39	39	39	37	44	44	40	39	64	38		31
TGF-β1	38	34	35	33	33	32	31	36	36	39	32	31	

significantly exceed the 11 changes between human and murine OP-1 (28). Diminished conservation across species is also found with BMP-3 and GDF-1 (10).

Comparison with OP-1—Alignments of human OP-2 with OP-1 (Fig. 3) may be used to decipher the structure and possible role of the pro-regions and to confirm the deduced sequences. OP-2 has only marginal homology with OP-1 in the signal peptide and adjacent pro-region (the first 33 residues). A nearby R*XX*R protease site of OP-1 is replaced in OP-2 by R*XX*G, at residue 37. However, a substitute R*XX*R pattern appears, shifted by 5 amino acids.

The pro-region of OP-2 and OP-1 share a potential glycosylation site (present also in BMP-5 and -6). While OP-1 lacks cysteines outside of the TGF-β domain, OP-2 contains a cysteine in the signal peptide and a second one at residue 31. A third cysteine is at residue 205 in the pro-region of human but not murine OP-2.

Proteolytic Maturation Sites in the Pro-domain—The proteolytic cleavages resulting in removal of pro-regions from the mature proteins occur immediately past the sequence R*XX*R in members of this family (7, 16, 28). Mutants of OP-1 with a minor alteration of the R*XX*R sequence are not properly cleaved.[2] Several genes of this family encode multiple R*XX*R patterns in the pro-region. For example, human OP-2 has three additional R*XX*R sites in the pro-region. N-terminal

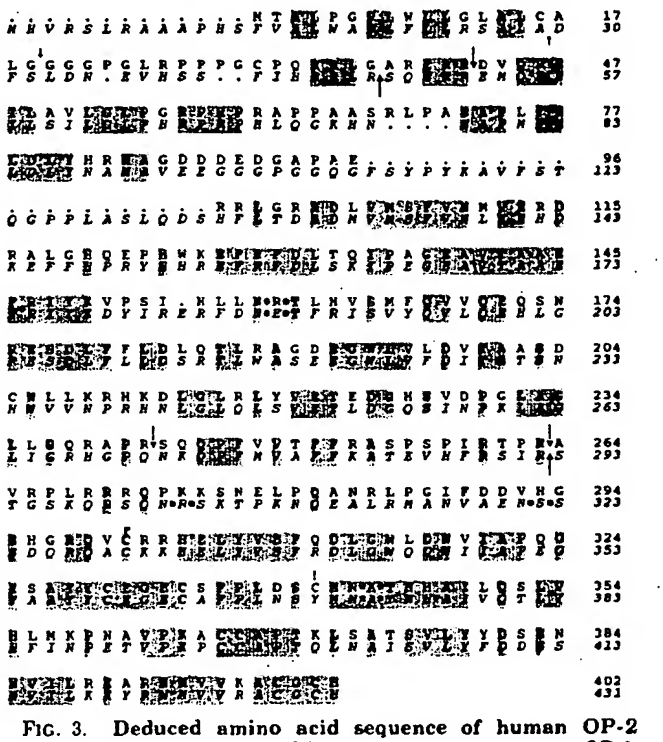

FIG. 3. **Deduced amino acid sequence of human OP-2 aligned with human OP-1.** The *upper sequence* repesents OP-2. OP-1 is shown in *italics*. *Shaded areas* indicate identical residues. The *dots* in the OP-1 and OP-2 sequences have been introduced for alignment. The signal peptidase cleavage sites are marked by *small arrows*. *Large arrows* mark the R*XX*R maturation sites. Potential *N*-glycosylation sites are marked by ✡. The first cysteine in the TGF-β domain is marked by a flag. The unique additional cysteine of OP-2 is marked by "!."

sequencing of mature OP-1 has shown the maturation site to be the most downstream R*XX*R pattern (25). However, additional upstream R*XX*R maturation sites can also be processed.[3] Fig. 4 shows alignments of mature N termini for different members of the TGF-β superfamily. Comparison of 16 examples shows that these R*XX*R sites are typically followed by serine or alanine. On occasion, R*XX*R is followed by aspartic acid, glycine, or glutamine.

N Termini of the Mature Proteins—An alignment of the

[2] Kaplan, P., Dorai, H., Özkaynak, E., and Oppermann, H., unpublished results.

[3] R. Tucker, personal communication.

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RKKRSTGVLLPLQ.....RSENRKRQRSGPHRRSQTLQFDEQTLRRARRRQNIEPRNC  BMP-3

RARRSQQPFVVTFFRASPSPIRTPRAVRPLRRRQPRRSNELPQANRLPGIFDDVHGSHGRQVC  OP-2
RSIRSTGSKQRSQNRSRTPRNQEALRMANVAENSSSDQRQAC  OP-1
RTTRSASSRRRQQSRNRSTQSQDVSRGSGSSDYNGSELETAC  Vgr-1
RSVRAAANKRRNQNRNRSSSHQDSSRMSSVGDYNTSEQRQAC  BMP-5

RSKRSASHPRRRRRSVSPNNVPLLEPMESTRSC  60A
RSIRDVSGGEGGGRGGRMKRHARRPTRRKNHDDTC  DPP

RHYRISRSLMQDEHSWSQIRPLLVTFGHDGKGHPLHK--RKRRQARK--RQNKHLRSSC  BMP-2
RIRRSLPQCSGNWAQLRPLLVTFGHDGRGHALTRRRRAKRSPKHHSQRARKKNKNC  BMP-4

RCKRPPKRLRSYSRLPFTASNIC  Vg-1
RPRRDAEPVLGGCPGGAC  GDF-1
RHRRALDTNYCFSSTEKNC  TGFB-1
RKRRALDAAYCFRNVQDNC  TGFB-2
RKRRALDTNYCFRNLEENC  TGFB-3
RKKRGVGQEYCFGNNGPHC  TGFB-5
RRRRDLDTDYCFGPGTDEKNC  TGFB-4
RRRRRGLECDGKVNIC  Activ.A
RIRKRGLECDGRTNLC  Activ.B
RARRSTPLMSWPWSPSALRLLQRPPEEPAAHANC  Inhib.a
RDPRGPGRAQRSAGAAAAADGPC  MIS
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FIG. 4. **N-terminal extensions of the mature proteins in the TGF-β superfamily.** The residues upstream of the 7-cysteine TGF-β domains are aligned in order of similarity. The *RXXR* proteolytic maturation sites followed by alanine or serine are *shaded*. Other *RXXR* patterns that do not quite conform are *underlined*. In several cases there are two potential maturation sites. The positively charged arginine and lysine are shown in *bold letters*.

mature N termini, up to the first cysteine of the TGF-β domain, for different superfamily members leads to interesting observations, regarding the length and composition (Fig. 4). Long N termini are found for the closely related OP-1, OP-2, and BMP-6 (37 aa) and BMP-5 (36 aa). N-terminal residues of BMP-5, BMP-6, and OP-1 differ mainly by conservative changes. The alignment of charged residues is nearly perfect for these proteins, and somewhat less so for OP-2. OP-2 lacks two potential glycosylation sites found in the mature N terminus of OP-1, BMP-5, and BMP-6.

Shorter N-terminal extensions (10–15 amino acids) are typical for the more distant members, including BMP-2, BMP-4, Vg-1, GDF-1, TGF-β 1–5, activin A, and MIS. BMP-3 is unique in having the *RXXR* site located 80 amino acids upstream of the TGF-β domain.

The mature N termini of OP-1, OP-2, BMP-5, BMP-6, BMP-2, BMP-4, BMP-3, and even DPP and 60A from *Drosophila*, share a high content of basic amino acid residues. This feature is found in all members of the superfamily that have osteogenic potential. It is absent in others, such as the TGF-β group, the activins, and MIS.

OP-2 mRNA Expression—In order to detect OP-2 expression we screened mRNA preparations from several organ tissues of 2-day-old rats, such as brain, calvaria, heart, kidney, and lung by Northern blot hybridization (Fig. 5). For this analysis brain was chosen since human OP-2 was isolated from a hippocampus cDNA library, calvaria were chosen as representative of bony tissue, and kidneys are a source of OP-1 mRNA (28). However, no OP-2 mRNA was detected in this analysis. As control we also probed with related genes in successive hybridizations and were able to detect mRNA of OP-1, Vgr-1, and BMP-4. OP-1 was found mainly in kidneys, Vgr-1 and BMP-4 mainly in lungs. A more detailed analysis is described below (see Fig. 7).

Since OP-2 cDNA was discovered in a mouse embryo cDNA library we then analyzed OP-2 mRNA expression in 8-, 10-, and 17-day mouse embryos as well as 6-day postnatal animals by Northern blot analysis of poly(A)$^+$ RNA. Extensive OP-2 mRNA expression was found in 8-day embryos, the message fell drastically in 10-day embryos, and was virtually absent in 17-day embryos (Fig. 6). The Northern result is consistent with the low abundance of OP-2 cDNA clones in a 17-day mouse embryo library (one clone in 10^6). Extremely low levels

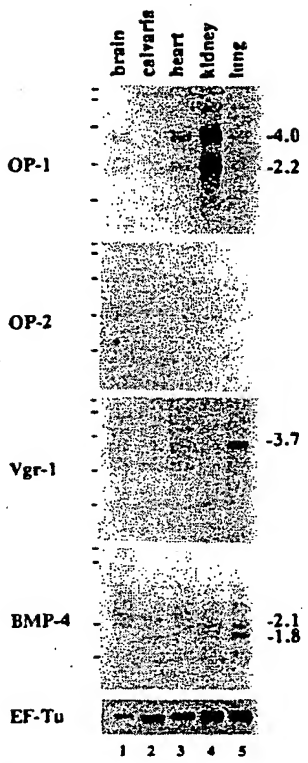

FIG. 5. **Analysis of OP-1, OP-2, Vgr-1, and BMP-4 mRNA in brain, calvaria, heart, kidneys, and lung of 2-day-old rats.** mRNA was purified (32) from several organs of Long-Evans rats. Equal amounts of poly(A)$^+$ RNA (10 μg) were loaded into each lane of a 1.2% agarose-formaldehyde gel. Sequential hybridizations were done with specific probes; murine OP-1 (0.7-kb *Bst*XI-*Bgl*I fragment), murine OP-2, 3′-untranslated region (0.42-kb *Xmn*I-*Hin*dIII fragment), murine Vgr-1 (0.26-kb *Pvu*II-*Sac*I fragment), and human BMP-4 (0.7-kb *Bst*EII fragment). The location of probes can also be seen in the line diagrams on the *right panel* of Fig. 7. Markings on the *right* of each hybridization correspond to 9.49-, 7.46-, 4.40-, 2.37-, and 1.35-kb RNA (0.24–9.49-kb RNA ladder; GIBCO-BRL).

were detected in 6-day postnatal animals and kidneys from 2-week-old animals.

mRNA Expression of Other TGF-β Superfamily Members in Specific Organs—In order to detect OP-2 expression in grown animals we screened several mouse organs by Northern hybridization. We extended this analysis to eight TGF-β-related genes (OP-1, OP-2, Vgr-1, BMP-2, BMP-3, BMP-4, BMP-5, and GDF-1) as no stringent or comprehensive study of mRNA expression is found in the literature. Cross-hybridization was minimized by using specific probes corresponding to highly diverged sequences. Poly(A)$^+$ RNA from brain, spleen, lung, heart, liver, and kidney of 2-week-old and 6–9-month-old male mice was analyzed on 1.2% agarose-formaldehyde gels, transferred to a membrane and hybridized sequentially to different probes (Fig. 7).

Clear results were obtained for most genes but no mRNA was detected in any organs studied for OP-2 (data not shown) and BMP-2. OP-1 message is mainly present in the kidneys and at a lower level in brain tissue as a total of four species (1.8, 2.2, 2.4, and 4.0 kb) (28). BMP-6/Vgr-1 mRNA, a single species of about 3.7 kb, is found in lungs and at low level in kidneys. BMP-5 mRNA is located in lungs and liver as a single species of 4.2 kb. BMP-4 mRNA is mainly in lungs and at much lower levels in kidneys (two species of 1.8 and 2.1 kb). BMP-4 specific signal was also detected in spleen and liver, however, migrating slightly different. Possibly this sig-

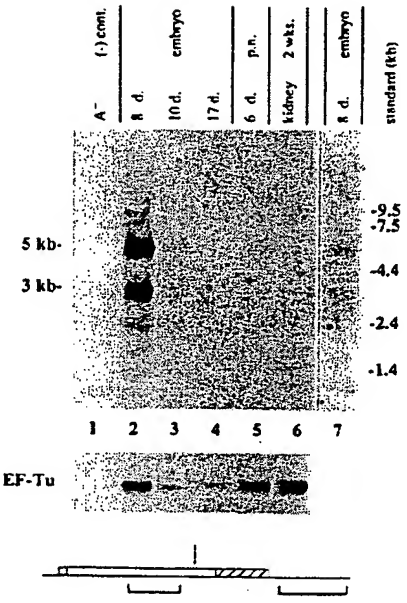

FIG. 6. **Expression of OP-2 mRNA in murine embryos and 6-day postnatal animal.** Equal amounts of poly(A)⁺ RNA (10 μg) were loaded into each lane. Hybridizations were done with probes specific for the pro-region of murine OP-2 (0.31-kb *Eco*RI-*Bam*HI fragment), and the 3′-untranslated region of murine OP-2 (0.42-kb *Xmn*I-*Hin*dIII fragment). The locations of the probes are shown with *bold lines* underneath the line diagram corresponding to the murine OP-2 cDNA. *Lane 1*, poly(A)⁺ kidney RNA (a negative control); *lanes 2–7*, poly(A)⁺ RNA obtained from 8-day embryos (*lane 2*), 10-day embryos (*lane 3*), 17-day embryos (*lane 4*), 6-day postnatal animals (*lane 5*), 2-week-old animal kidneys (*lane 6*), 8-day embryos (*lane 7*). *Lanes 1–6*, hybridization to a pro-region specific OP-2 probe, and *lane 7*, hybridization to a 3′-untranslated region specific OP-2 probe. The *gray box* in the OP-2 line diagram indicates the signal peptide and the *hatched box* corresponds to the TGF-β domain. An *arrow* marks the location of the maturation site. The sizes of the major OP-2 mRNA species are shown on the *left*.

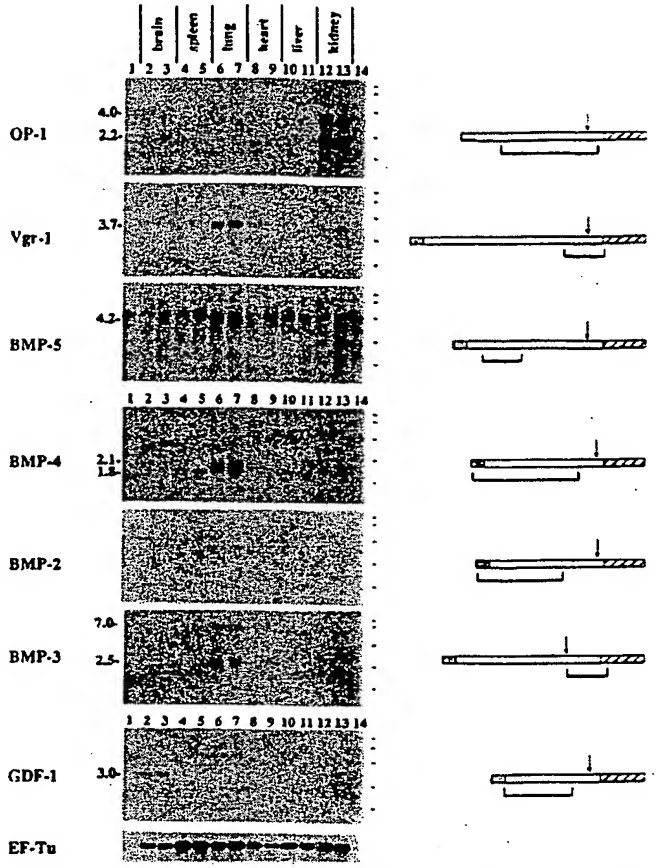

FIG. 7. **mRNA expression of TGF-β superfamily members in organs of mice.** Expression of OP-1, Vgr-1, BMP-5, BMP-4, BMP-2, BMP-3, and GDF-1 mRNA was studied in selected organ tissues of 2-week- and 6–9-month-old mice. Equal amounts (5 μg) of poly(A)⁺ RNA were loaded onto each lane of a 1.2% agarose-formaldehyde gel. *Lanes 2, 4, 6, 8, 10,* and *12* correspond to poly(A)⁺ RNA obtained from 2-week-old mice, *lanes 3, 5, 7, 9, 11,* and *13* correspond to poly(A)⁺ RNA obtained from 6–9-month-old mice. *Lanes 1* and *14* contain poly(A)⁻ RNA (from brain and liver of 2-week-old mice). The same membrane was used for sequential hybridizations with probes specific for: murine OP-1 (0.7-kb *Bst*X1-*Bgl*I fragment); murine Vgr-1 (0.26-kb *Pvu*II-*Sac*I fragment); murine BMP-5 (0.28-kb *Pst*I fragment, *Pst*I sites were introduced during the PCR); human BMP-4 (0.7-kb *Bst*EII fragment); murine BMP-3 (0.27-kb *Eco*RI-*Eco*RV fragment); human BMP-2 (0.7-kb *Nco*I fragment); murine GDF-1 (0.47-kb *Cla*I-*Pst*I fragment, *Cla*I site from the vector); murine EF-Tu (0.5-kb *Hin*dIII-*Bam*HI fragment, *Bam*HI from the vector). Locations of the probes are shown in *bold lines* underneath the cDNA diagrams. The *gray boxes* in the diagrams indicate the signal peptides and *hatched boxes* correspond to the 7-cysteine/TGF-β-domains. *Arrows* mark the location of maturation sites. The sizes of the major RNA species are shown on the left. Markings on the right of each hybridization correspond to 9.49-, 7.46-, 4.40-, 2.37-, and 1.35-kb RNA (0.24–9.49-kb RNA ladder).

nal results from cross-hybridization with an unknown BMP-4 related gene. BMP-3 mRNA is observed in lungs (two species of 2.5 and 7 kb and a minor species of 3.2 kb). GDF-1-specific message of 3 kb is found only in brain, in accordance with Lee (10).

Age-dependent mRNA Expression—An age-dependent mRNA expression is not only observed for OP-2 but also with related genes: OP-1 mRNA in the kidneys of young animals (2 weeks old) is approximately twice the level of adult animals (6–9 months old) (Fig. 7). Similarly, BMP-5 mRNA is detected in the lungs and liver of young animals, much more so than in adult animals. For Vgr-1 and BMP-4, a reverse pattern of age-dependent expression is observed in the lungs, with levels approximately three times higher in adult over young animals. BMP-3 and GDF-1 mRNA levels in lungs and brain remained unchanged with age.

Presence of Multiple or Large Transcripts—The presence of oversized and multiple mRNA transcripts of OP-2, two species of 3 and 5 kb in 8-day embryos, has ample precedence among the other related genes. Most members of the superfamily have transcripts much larger than the expected 1.4–1.8-kb size and several have different size transcripts (Fig. 7). Northern blots showed four OP-1 mRNA species: 1.8, 2.2, 2.4, and 4 kb (28). BMP-3 also has multiple size transcripts (2.5 and 7.0 kb). BMP-5 and Vgr-1 (8) have single size transcripts of about 4 kb, much longer than expected.

Genomic Structure of the Human OP-2 Gene—The human OP-2 locus was isolated from a genomic library on three overlapping phage clones (λ O34, O35, O28), the OP-2 coding information spread over 7 exons and 27 kb. The entire nucleotide sequence with exception of the largest two introns, 1 and 3, was determined from 20 subclones (Fig. 8). The exon-intron boundaries are shown in Fig. 9. A comparison of exon-intron boundaries in the TGF-β domain showed matching locations with those of OP-1 (9). The first OP-2 exon contains 334 bp of coding sequence (111 amino acids), including the signal peptide, and is followed by the largest intron (14.6 kb). The second exon (190 bp, 64 amino acids) is separated by a short intron (0.4 kb) from exon 3 (149 bp, 49 amino acids). It follows a large third intron of 9.5 kb. The fourth exon (195

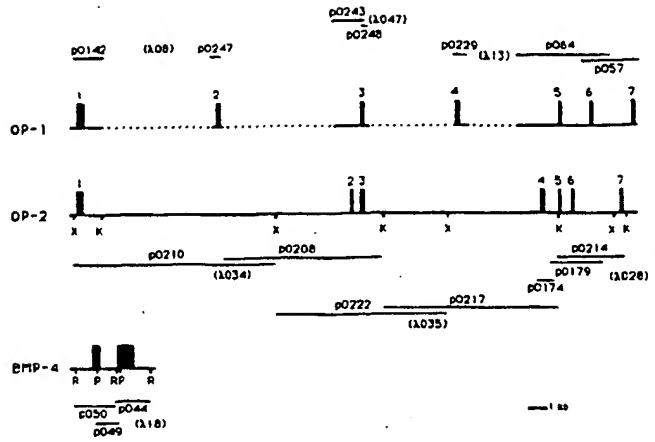

FIG. 8. **Genomic structure of OP-1, OP-2, and BMP-4.** OP-1 and OP-2 genes contain seven exons, whereas the BMP-4 gene contains two exons. Some subclones used to derive the genomic structures are shown in *bold lines*, with the corresponding phage clones in *parentheses*. The OP-2 gene spans 27 kb of genomic distance (contained in three phage clones). *K* and *X* indicate *Kpn*I and *Xho*I sites, respectively. *R* and *P* indicate *Eco*RI and *Pst*I sites in the BMP-4 locus. *Dotted lines* in the OP-1 diagram indicate undetermined intron lengths. Genomic subclones used to determine the OP-1 structure: pO142 (1.7-kb *Pst*I fragment), pO247 (0.6-kb *Hae*III fragment), pO243 (1.7-kb *Pst*I fragment), pO248 (0.3-kb *Sau*3AI fragment), pO229 (0.7-kb *Pst*I fragment), pO64 (5-kb *Eco*R1 fragment), pO57 (3-kb *Bam*HI fragment). Genomic subclones used to determine the OP-2 structure: pO210 (11-kb *Xho*I fragment), pO208 (8.4-kb *Bam*HI-*Kpn*I fragment), pO222 (9-kb *Xho*I fragment), pO217 (9.4-kb *Kpn*I fragment), pO174 (0.8-kb *Pst*I fragment), pO179 (2.8-kb *Stu*I fragment), pO214 (3.6-kb *Kpn*I fragment). Genomic subclones used to determine the BMP-4 structure: pO50 (2.2-kb *Eco*RI fragment), pO49 (1.2-kb *Pst*I fragment), pO44 (1.9-kb *Eco*RI fragment).

bp, 65 amino acids) encodes the RTPR↓A maturation site and is followed by a 0.8-kb intron. The TGF-β domain resides on exons 5–7: exon 5 (80 bp, 27 amino acids) encodes the first cysteine of mature OP-2 and is followed by intron 5 (0.5 kb in length), exon 6 (111 bp, 37 amino acids) is separated by a 2.5-kb intron from the seventh, last exon with 147 bp (49 amino acids) of coding sequence.

Genomic Structure of Human OP-1—To complete the genomic comparison of OP-2 and OP-1 we isolated the remaining exons of OP-1 from a human genomic library on three phage clones: exon 1 and 2 (λ O8), exon 3 (λ O47), and exon 4 (λ O37). The structure of OP-1, with exception of the first four introns was determined as follows. Exon 1 contains 418 bp of coding sequence (139 amino acids), and exon 2 is 193 bp (65 amino acids). Exon 3 measures 148 bp (49 amino acids) and exon 4 is 198 bp (66 amino acids) in length. Exon 5, 78 bp (26 amino acids), is followed by a 1.6-kb intron. Exon 6 is 111 bp (37 amino acids), followed by a 2.1-kb intron. The last OP-1 exon contains 147 bp of coding sequence (49 amino acids).

Compared with OP-2, all OP-1 splice junctions are also located at identical positions (Fig. 8), indicating that OP-1 and OP-2 diverged more recently than BMP-4.

Genomic Structure of Human BMP-4—Screening of a human genomic library with a consensus gene probe (9), resulted in isolation of the entire genomic BMP-4 in one clone (λ 18) on two *Eco*RI fragments (pO50, pO44). The complete BMP-4 coding region is present in only two exons, 0.37 and 0.86 kb, interrupted by an intron of 0.96 kb (Fig. 8).

DISCUSSION

The present study describes a new gene which is closely related to OP-1, BMP-5, Vgr-1, and *Drosophila* 60A. Based on the extensive homology with other osteogenic/bone morphogenetic proteins that are active in the rat subcutaneous implant assay, it is expected that OP-2 has osteogenic potential. The genomic structures of OP-1 and OP-2 both contain seven exons, spread over large distance with matching exon-intron boundaries, thus setting them apart from from BMP-2, BMP-4, and BMP-3.

OP-2 is uniquely marked by an 8th cysteine in the "7-cysteine domain" of the TGF-β superfamily. Since the mature members of the TGF-β superfamily are dimeric, this extra cysteine may participate in and stabilize the dimer formation. Recently the crystal structure of TGF-β has been published (34, 35), and a single intermolecular disulfide bridge has been identified. Substitution of the respective cysteine in OP-2 by serine, for example, by site-directed mutagenesis might show whether the additional cysteine can preserve the dimer structure.

FIG. 9. **Exon-intron boundaries of OP-2.** The partial nucleotide sequences of the human OP-2 gene show all 12 exon-intron boundaries. The intron sequences are shown in *lowercase lettering*. The maturation cleavage site is marked with an *arrowhead*, and the first cysteine in the TGF-β domain is *circled*.

A close inspection of the proteolytic maturation sites of several precursors shows that the pattern RXXR can be further specified as RXXR↓A or RXXR↓S, which are found in the majority of cases. In OP-2 the TGF-β domain follows two potential maturation sites, 21 residues apart. Multiple RXXR sites near the TGF-β domain are also seen in BMP-2 and BMP-4, about 30 residues apart. Additional proteolytic maturation sites are found further upstream in the pro-region. In human OP-2 the multiple maturation sites could release five potential polypeptides, spanning from amino acid 20 (signal peptide cleavage site) to aa 42, from 43 to 101, from 102 to 242, from 243 to 263, and the mature C-terminal domain, with the possibility of additional biological activities.

The mature N termini of different members of the TGF-β superfamily are quite diverse in their amino acid sequence. The extensive variations found in the mature N termini may provide distinction between the otherwise conserved mature proteins. Possibly this region has diverged because it is not crucial for receptor binding or protein folding. However, for a given protein this region is still relatively well conserved among animal species, in support of a functional role. N-terminal sequences may supply the individual proteins with more specific recognition for their respective receptors. They may allow targeting of specific tissues via binding to extracellular matrix. The N terminus of OP-2 has some similarity in composition to nuclear localization sequences that contain several prolines interspersed with lysines or arginines (36), a feature not seen for the other osteogenic proteins.

The high content of basic amino acids in the N terminus is characteristic for the osteogenic proteins. In contrast, a mature N terminus of only 10–14 amino acids with few basic residues is characteristic for the more distantly related proteins, TGF-β 1–5, the activins, inhibin α, and MIS. The positively charged N termini of the osteogenic proteins may permit binding of the proteins to hydroxylapatite, a resin traditionally used during purification of the osteogenic protein (29). *In vivo*, the basic N termini may mediate the deposition of osteogenic proteins in bony tissue. However, in the subcutaneous bone induction assay the N termini are not essential for biological activity (25) perhaps due to the local administration of the protein, immobilized on collagen matrix. The insect proteins, DPP and 60A, also display positively charged N termini, even though their role is not bone induction.

The analysis of mRNA species for osteogenic proteins has been difficult due to the extremely low level of natural expression of these proteins, and published data are sparse. Analysis by dot blot or by *in situ* hybridization is confounded by the possibility of cross hybridization of probes to different members. In our study we have minimized this possibility by careful choice of probes. Selection of poly(A)$^+$ RNA on oligo(dT)-cellulose is necessary due to low levels of expression of these mRNAs. The low level of expression may be a reflection of regulatory function and high biological activity. Nanogram amounts suffice for subcutaneous bone induction in the rat model (29).

Northern analysis of several organs with probes for various family members indicates that mRNA for most of them is expressed in specific organs. OP-1 mRNA is expressed mainly in kidneys and bladder (28), which may explain the epithelial osteogenesis, discovered by Huggins over 60 years ago. Huggins (38) noted that urinary tract epithelia implanted into the abdominal wall of dogs evoked large amounts of bone within 12 days. OP-1 mRNA is also expressed in brain which is the sole site of GDF-1 expression. In contrast, we find BMP-3, BMP-4, BMP-5, and BMP-6/Vgr-1 to be primarily expressed in the lungs. The lungs may participate in the growth regulation of bone and connective tissues in an endocrine manner, as proposed for the kidney (28).

OP-2- and BMP-2-specific mRNAs were not detected in any of the adult organs by our Northern hybridization analysis. OP-2 cDNA was found at low abundance in a hippocampus cDNA library and may be expressed at low levels in brain. However, OP-2 mRNA was found at relatively high levels in 8-day mouse embryos, indicating a developmental role; in the adult animal OP-2 and BMP-2 may be expressed in a more discrete location, or primarily during tissue regeneration. While the timing of expression for BMP-5 and OP-1 seems to be directly related to growth, an inverse relationship was found for Vgr-1 and BMP-4. The level of BMP-3 expression (in lungs) did not change with the age of the animal.

The early embryo displays two oversize OP-2 mRNA species. Hence, the OP-2 locus may be considerably larger that the 27 kb cloned so far. The multiple size transcripts observed for OP-1, OP-2, BMP-3, and BMP-4 may result from splicing events that affect coding or untranslated regions, or they may represent bicistronic mRNA species (10). Diverse transcripts have also been seen with DPP. These multiple DPP-specific mRNAs have been shown to be due to alternatively spliced 5′-untranslated exons (37).

There is an apparent redundancy of osteogenic proteins in the TGF-β superfamily. Since bone has different architectures and fine structures depending on the anatomical localization (for example, long bones, facial bones, skull plates, and dentin), the osteogenic proteins may have evolved along with the different types of bone. Roles other than bone formation are likely since analogs of the osteogenic proteins have been found in invertebrates (DPP and 60A of *Drosophila*). Multiple functions are indicated by the fact that the bone morphogenetic proteins are expressed in early embryonic development as well as later in life.

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REFERENCES

1. Rebagliati, M. R., Weeks, D. L., Harvey, R. P., and Melton, D. A. (1985) *Cell* **42**, 769–777
2. Cate, R. L., Mattaliano, R. J., Hession, C., Tizard, R., Farber, N. M., Cheung, A., Ninfa, E. G., Frey, A. Z., Gash, D. J., Chow, E. P., Fisher, R. A., Bertonis, J. M., Torres, G., Wallner, B. P., Ramachandran, K. L., Ragin, R. C., Manganaro, T. F., MacLaughlin, D. T., and Donahoe, P. K. (1986) *Cell* **45**, 685–698
3. Mason, A. J., Hayflick, J. S., Ling, N., Esch, F., Ueno, N., Ying, S-Y., Guillemin, R., Niall, H., and Seeburg, P. H. (1985) *Nature* **318**, 659–663
4. Ling, N., Ying, S-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. (1986) *Nature* **321**, 779–782
5. Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. (1986) *Nature* **321**, 776–779
6. Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988) *Science* **242**, 1528–1533
7. Celeste, A. J., Iannazzi, J. A., Taylor, R. C., Hewick, R. M., Rosen, V., Wang, E. A., and Wozney, J. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 9843–9847
8. Lyons, K., Graycar, J. L., Lee, A., Hashmi, S., Lindquist, P. B., Chen, E. Y., Hogan, B. L. M., and Derynck, R. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 4554–4558
9. Özkaynak, E., Rueger, D. C., Drier, E. A., Corbett, C., Ridge, R. J., Sampath, T. K., and Oppermann, H. (1990) *EMBO J.* **9**, 2085–2093
10. Lee, S-J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 4250–4254
11. Segal, D., and Gelbart, W. M. (1985) *Genetics* **109**, 119–143
12. Wharton, K. A., Thomsen, G. H., and Gelbart, W. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9214–9218
13. Mathews, L. S., and Vale, W. W. (1991) *Cell* **65**, 973–982
14. Lin, H. Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R., and Lodish, H. F. (1992) *Cell* **68**, 775–785
15. Massagué, J. (1992) *Cell* **69**, 1067–1070
16. Panganiban, G. E. F., Rashka, K. E., Neitzel, M. D., and Hoffmann, F. M. (1990) *Mol. Cell. Biol.* **10**, 2669–2677
17. Irish, V. F., and Gelbart, W. M. (1987) *Genes & Dev.* **1**, 868–879
18. Van den Eijnden-Van Raaij, A. J. M., van Zoelent, E. J. J., van Nimmen, K., Koster, C. H., Snoek, G. T., Durston, A. J., and Huylebroeck, D. (1990) *Nature* **345**, 732–734
19. Smith, J. C., Price, B. M. J., van Nimmen, K., and Huylebroeck, D. (1990) *Nature* **345**, 729–731

20. Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W., and Melton, D. A. (1990) *Cell* **63**, 485–493
21. Köster, M., Plessow, S., Clement, J. H., Lorenz, A., Tiedemann, H., and Knöchel, W. (1991) *Mech. Dev.* **33**, 191–200
22. Behringer, R. R., Cate, R. L., Froelick, G. J., Palmiter, R. D., and Brinster, R. L. (1990) *Nature* **345**, 167–170
23. Wang, E. A., Rosen, V., D'Alessandro, J. S., Bauduy, M., Cordes, P., Harada, T., Israel, D., Hewick, R. M., Kerns, K. M., Lapan, P., Luxenberg, D. P., McQuaid, D., Mousatsos, I. K., Nove, J., and Wozney, J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2220–2224
24. Hammonds, R. G. Jr, Schwall, R., Dudley, A., Berkemeier, L., Lai, C., Lee, J., Cunningham, N., Reddi, A. H., Wood, W. I., and Mason, A. J. (1991) *Mol. Endocrinol.* **5**, 149–155
25. Sampath, T. K., Maliakal, J. C., Hauschka, P. V., Jones, W. K., Sasak, H., Tucker, R. F., White, K., Coughlin, J. E., Tucker, M. M., Pang, R. H. L., Corbett, C., Özkaynak, E., Oppermann, H., and Rueger, D. C. (1992) *J. Biol. Chem.* **267**, 20352–20362
26. Sampath, T. K., and Reddi, A. H. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 7599–7603
27. Cook, S. D., Baffes, G. C., Sampath, T. K., and Rueger, D. C. (1991) *J. Mineral Res.* **6**, Suppl. 1 (Abstr. 283)
28. Özkaynak, E., Schnegelsberg, P. N. J., and Oppermann, H. (1991) *Biochem. Biophys. Res. Commun.* **179**, 116–123
29. Sampath, T. K., Coughlin, J. E., Whetstone, R. M., Banach, D., Corbett, C., Ridge, R. J., Özkaynak, E., Oppermann, H., and Rueger, D. C. (1990) *J. Biol. Chem.* **265**, 13198–13205
30. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
31. Özkaynak, E., and Putney, S. D. (1987) *Bio/Techniques* **5**, 770–773
32. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
33. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (eds) (1990) *Current Protocols in Molecular Biology*, Wiley-Interscience, New York
34. Daopin, S., Piez, K. A., Ogawa, Y., and Davies, D. R. (1992) *Science* **257**, 369–373
35. Schlunegger, M. P., and Gruetter, M. G. (1992) *Nature* **358**, 430–434
36. Wagner, P., Kunz, J., Koller, A., and Hall, M. N. (1990) *FEBS Lett.* **275**, 1–5
37. St. Johnston, R. D., Hoffmann, F. M., Blackman, R. K., Segal, D., Grimaila, R., Padgett, R. W., Irick, H. A., and Gelbart, W. M. (1990) *Genes & Dev.* **4**, 1114–1127
38. Huggins, C. B. (1931) *Arch. Surg.* **22**, 377–408